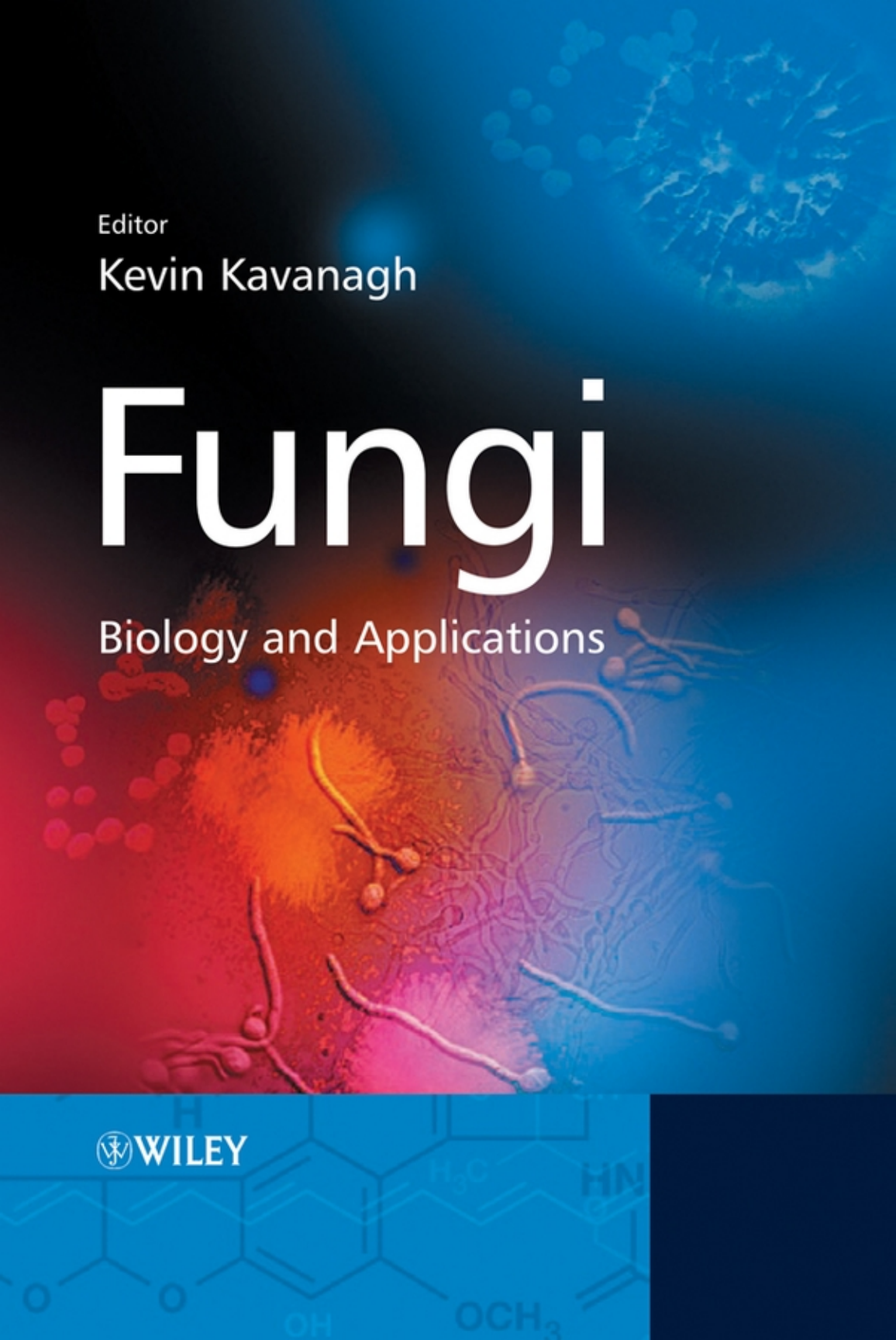
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Editor

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Preface

Fungi make an enormous contribution to our life. The role of yeast in the production of alcohol and bread is well characterized. We consume fungi directly in the form of edible mushrooms and in 'blue cheeses' which get their characteristic flavour and aroma from the presence of fungi. Fungi are also used for the production of antibiotics, such as penicillin, and enzymes for use in the food industry. Over the last three decades fungi have been utilized for the production of recombinant ('foreign') proteins, some of which have great therapeutic potential. Although infrequently recognized as important decomposers of organic detritus, fungi play a significant role in degrading biological matter, such as fallen leaves. On a more negative note some fungi (for example members of the genus *Candida* or *Aspergillus*) are capable of causing serious life-threatening infections in immuno-compromised patients, and other fungi can be serious environmental contaminants.

The aim of this book is to provide a detailed description of the biology, biotechnological applications and medical significance of fungi. The book commences with an in-depth description of the physiology of fungi in which the structure, metabolism and growth of fungi are described. This is followed by a chapter dedicated to the genetics of fungi. In this chapter the life cycles of a number of representative fungi are described and the use of fungi for genetic analysis is outlined. The advent of genomics and proteomics has revolutionized our study of the cell. Chapter 3 describes how genomics, transcriptomics and proteomics have increased our knowledge of fungi and made available new opportunities for exploiting fungi for the good of humanity. Chapter 4 describes the main fermentation systems used with fungi and how these can be exploited to produce a range of commercially valuable products. Chapter 5 gives an overview of how fungi are utilized for producing antibiotics, enzymes and a range of chemical products such as citric acid. Chapter 6 focuses on the exploitation of fungi for the production of heterologous proteins and illustrates how yeast can be used for the production of hepatitis B antigens. The medical con-

ditions caused by pathogenic fungi are outlined in Chapter 7, which shows the main classes of pathogenic fungi and the types of condition that predispose the host to infection. The range of antifungal drugs used to combat fungal infections is described in Chapter 8 and the requirement for new classes of antifungal with distinct mode(s) of action is outlined. The final chapter describes the main fungal pathogens of plants and assesses the impact of such pathogens on the global supply of food.

This book gives a comprehensive introduction to fungi in terms of their biology, genetics, medical significance and biotechnological potential. Each chapter is written by internationally recognized experts so the reader is given an up-to-date and detailed account of our knowledge of the biology and various applications of fungi.

Kevin Kavanagh

1

Introduction to Fungal Physiology

Graeme M. Walker and Nia A. White

1.1 Introduction

Fungal physiology refers to the nutrition, metabolism, growth, reproduction and death of fungal cells. It also generally relates to interaction of fungi with their biotic and abiotic environment, including cellular responses to stress. The physiology of fungal cells impacts significantly on the environment, industry and human health. In relation to ecological aspects, the biogeochemical cycling of elements in Nature would not be possible without the participation of fungi as primary decomposers of organic material. Furthermore, the dynamics of fungal activities are central to the efficiency of forestry and agricultural operations, as mutualistic symbionts, pathogens and saprophytes, by mobilizing nutrients and affecting the physico-chemical environment. Fungal metabolism is also responsible for detoxification of organic pollutants and for bioremediation of heavy metals in the environment. The production of many economically important industrial commodities relies on exploitation of yeast and fungal metabolism and these include such diverse products as whole foods, food additives, fermented beverages, antibiotics, pigments, pharmaceuticals, biofuels, industrial enzymes, vitamins, organic and fatty acids and sterols. In terms of human health, some yeasts and fungi represent major opportunistic life-threatening pathogens, whilst others are life-savers as they provide antimicrobial and chemotherapeutic agents. In modern biotechnology, several yeast species are being exploited as ideal hosts for the expression of human therapeutic proteins following recombinant DNA technology. In addition to the direct industrial exploitation of

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yeasts and fungi, it is important to note that these organisms, most notably the yeast *Saccharomyces cerevisiae*, play increasingly significant roles as model eukaryotic cells in furthering our fundamental knowledge of biological and biomedical science. This is especially the case now that several fungal genomes, including that of *S. cerevisiae*, have been completely sequenced and the information gleaned from fungal genomics and proteomics is providing valuable insight into human genetics and heritable disorders. However, knowledge of cell physiology is essential if the functions of many of the currently unknown fungal genes are to be fully elucidated.

It is apparent, therefore, that fungi are important organisms for human society, health and well-being and that studies of fungal physiology are very pertinent to our understanding, control and exploitation of this group of microorganisms. This chapter describes some basic aspects of fungal cell physiology, focusing primarily on nutrition, growth and metabolism in unicellular yeasts and filamentous fungi.

1.2 Morphology of yeasts and fungi

Most fungi are filamentous, many grow as unicellular yeasts and some primitive fungi such as the chytridomycetes grow as individual rounded cells or dichotomous branched chains of cells with rootlike rhizoids for attachment to a nutrient resource. Here we will consider the most common growth forms, the filamentous fungi and unicellular yeasts.

1.2.1 Filamentous fungi

The gross morphologies of macrofungi and microfungi are incredibly diverse (see Plate 1). For example, we can easily recognize a variety of mushrooms and toadstools, the sexual fruiting bodies of certain macrofungi (the higher fungi Asomycotina and Basidiomycotina and related forms), during a walk through pasture or woodland. Microfungi too are diverse; some commonly referred to as moulds are often observed on decaying foods and detritus, whereas many, including the coloured rusts, smuts and mildews, are regular plant pathogens. Closer inspection of these visible structures, however, reveals that all are composed of aggregated long, branching threads termed hyphae (singular hypha), organized to support spores for reproduction and dissemination. The hyphae of these aerial structures extend and branch within the supporting substratum as a network, termed a mycelium, from which the apically growing hyphae seek out, exploit and translocate available nutrients. Apically growing hyphae usually have a relatively constant diameter ranging from 1 to 30 μm or more, depending on species and growth conditions. Filamentous fungi may be cultivated within the laboratory on a variety of different liquid or solid media. On agar,

the radially expanding colonial growth form of the fungal mycelium is most evident, extending from an inoculum, on, within and sometimes above the substrate, forming a near-spherical three-dimensional colony. This radiating, circular pattern is also visible during the growth of fairy ring fungi in grassland and as ringworm infections of the skin.

The hyphae of individual fungi may (theoretically) extend endlessly via apical growth, provided they are supported with appropriate nutrients and other environmental conditions. Eucarpic fungi are therefore spatially and temporally indeterminate organisms and, unlike animal, plant and other microbial individuals, have no predetermined maximum size or age. The mycelium is not, however, simply a homogeneously extending entity, but displays considerable developmental plasticity. Different interconnected regions of the fungal mycelium may grow, branch, anastomose (fuse), age, die, sporulate and display varying physiological and biochemical activities at different times or even simultaneously, depending on local micro-environmental conditions. Thus, colonies growing on relatively homogeneous media may be pigmented, exhibit different morphological sectors, produce aerial structures, grow as fast–effuse or slow–dense forms and even exhibit rhythmic growth (Plate 1). As well as reproductive structures and substrate mycelium, certain higher fungi, most notably the basidiomycetes, when growing within an environment where nutrients are distributed heterogeneously, can differentiate into long stringlike structures called rhizomorphs or cords. These linear organs have evolved to explore rapidly for, connect and translocate water and nutrients between patches of resource (e.g. pieces of fallen wood on the forest floor or from tree root to tree root). Accordingly, many, particularly mature rhizomorphs, contain internal vessel hyphae that possess a wide diameter, forming a channel running along the organ. The peripheral hyphae are often closely packed and melanized for insulation.

Filamentous fungi and yeasts are simply different styles of fungal growth suitable for occupation of different habitats and produced by differing cell growth polarities. Many species termed dimorphic fungi can adopt either the hyphal or unicellular yeast forms according to environmental circumstances, e.g certain important human and animal pathogens (yeast forms mobilized in body fluids, hyphal forms for tissue invasion).

1.2.2 Yeasts

Yeasts are unicellular (mostly Ascomycete, Basidiomycete or Deuteromycete) fungi that divide asexually by budding or fission and whose individual cell size can vary widely from 2–3 μm to 20–50 μm in length and 1–10 μm in width. *S. cerevisiae*, the best known yeast, is generally ellipsoid in shape with a large diameter of 5–10 μm and a small diameter of 1–7 μm (Figure 1.1).

The morphologies of agar-grown yeasts show great diversity in terms of colour, texture and geometry (peripheries, contours) of giant colonies. Table 1.1 shows the diversity of cell shapes found in the yeasts. Several yeasts are pig-

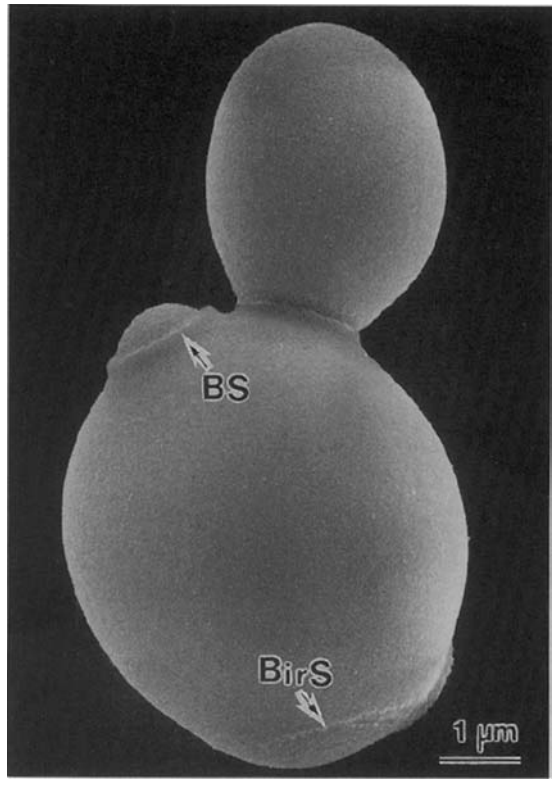

Figure 1.1 Scanning electron micrograph of a typical yeast cell (×10 000) – BS, bud scar; BirS, birth scar (Reproduced with kind permission of Professor Masako Osumi, Japan Women's University, Tokyo)

mented and the following colours may be visualized in surface-grown colonies: cream (e.g. *S. cerevisiae*); white (e.g. *Geotrichum candidum*); black (e.g. *Aureobasidium pullulans*); pink (e.g. *Phaffia rhodozyma*); red (e.g. *Rhodotorula rubra*); orange (e.g. *Rhodosporidium* spp.) and yellow (e.g. *Cryptococcus laurentii*). The pigments of some yeasts have biotechnological uses, including astaxanthin from *P. rhodozyma* in aquacultural feed supplements for farmed salmon (that are unable to synthesize these natural pink compounds).

1.3 Ultrastructure and function of fungal cells

1.3.1 The fungal cell surface

The cell envelope in yeasts and fungi is the peripheral structure that encases the cytoplasm and comprises the plasma membrane, the periplasm, the cell wall and additional extracellular structural components (such as fimbriae and capsules).

Table 1.1 Diversity of yeast cell shapes

Cell shape	Description	Examples of yeast genera
Ellipsoid	Ovoid shaped cells	*Saccharomyces*
Cylindrical	Elongated cells with hemispherical ends	*Schizosaccharomyces*
Apiculate	Lemon shaped	*Hanseniaspora, Saccharomycodes*
Ogival	Elongated cell rounded at one end and pointed at other	*Dekkera, Brettanomyces*
Flask shaped	Cells dividing by bud fission	*Pityrosporum*
Miscellaneous shapes	Triangular Curved Spherical Stalked	*Trigonopsis* *Cryptococcus* (e.g. *C. cereanus*) *Debaryomyces* *Sterigmatomyces*
Pseudohyphal	Chains of budding yeast cells that have elongated without detachment	*Candida* (e.g. *C. albicans*)
Hyphal	Branched or unbranched filamentous cells which form from germ tubes. Septa may be laid down by the continuously extending hyphal tip. Hyphae may give rise to blastospores	*Candida albicans*
Dimorphic	Yeasts that grow vegetatively in either yeast or filamentous (hyphal or pseudohyphal) form	*Candida albicans* *Saccharomycopsis fibuligera* *Kluyveromyces marxianus* *Malassezia furfur* *Yarrowia lipolytica* *Histoplasma capsulatum*

The cell wall represents a dynamically forming exoskeleton that protects the fungal protoplast from the external environment and defines growth, cellular strength, shape and interactive properties. In filamentous fungi, cell wall formation and organization is intimately bound, to the process of apical growth. Thus, for example, in *Neurospora crassa* the wall is thin (approximately 50 nm) at the apex but becomes thicker (approximately 125 nm) 250 μm behind the tip. The plasma membrane component of the fungal cell envelope is a phospholipid bilayer interspersed with globular proteins that dictates entry of nutrients and exit of metabolites and represents a selective barrier for their translocation. Ergosterol is the major sterol found in the membranes of fungi, in contrast to

the cholesterol found in the membranes of animals and phytosterols in plants. This distinction is exploited during the use of certain antifungal agents used to treat some fungal infections and can be used as an assay tool to quantify fungal growth. The periplasm, or periplasmic space, is the region external to the plasma membrane and internal to the cell wall. In yeast cells, it comprises secreted proteins (mannoproteins) and enzymes (such as invertase and acid phosphatase) that are unable to traverse the cell wall. In filamentous fungi, the cell membrane and wall may be intimately bound, as hyphae are often resistant to plasmolysis.

Fungal cell surface topological features can be visualized using scanning electron microscopy (SEM) and nanometre resolution achieved using atomic force microscopy (AFM). The latter is beneficial as it can be employed with unfixed, living cells and avoids potentially misleading artefacts that may arise when preparing cells for electron microscopy.

Ultrastructural analysis of fungal cell walls reveals a thick, complex fibrillar network. The cell walls of filamentous fungi are mainly composed of different polysaccharides according to taxonomic group (either chitin, glucans, mannoproteins, chitosan, chitin, polyglucuronic acid or cellulose), together with smaller quantities of proteins and glycoproteins (Table 1.2). Generally, the semicrystalline microfibrillar components are organized in a network mainly in the central cell wall region and are embedded within an amorphous matrix. Bonding occurs between certain components behind the extending hyphal tip, thereby strengthening the entire wall structure. There is evidence to suggest that the cell wall is a dynamic structure where considerable quantitative and qualitative differences occur not only between different fungal species, but also between different morphological forms of the same species and even in response to

Table 1.2 The major polymers found in different taxonomical groups of fungi together with the presence of perforate septa in these groups (adapted from Deacon, 2000; Carlile, Watkinson and Gooday, 2001)

Taxonomic grouping	Fibrillar polymers	Matrix polymers	Perforate septa present or absent
Oomycetes	β(1,3), β(1,6)-glucan cellulose	Glucan	Absent
Chytridomycetes	Chitin; glucan	Glucan	Absent
Zygomycetes	Chitin; chitosan	Polyglucuronic acid; glucuronomannoproteins	Absent
Basidiomycetes	Citin; β(1,3)-β(1,6) glucans	α(1,3)-glucan; xylomannoproteins	Present (mostly Dolipore)
Ascomycetes/ deuteromycetes	Citin; β(1,3)-β(1,6) glucans	α(1,3)-glucan; galactomannoproteins	Present (mostly simple with large central pore)

environmental stress. For example, a class of hydrophobic proteins called hydrophobins are localized within the aerial growth or appresoria (terminal swellings involved in infection) of certain fungi, whereas pigmented melanins are often found within some fungal cell walls to insulate against biotic and abiotic stresses.

The hyphae of higher fungi extend via tip growth followed by cross-wall formation or septation, whereas the lower fungi remain aseptate (apart from to segregate spores or damaged colony regions). Septa may offer some structural support to hyphae. Significantly, septa serve to compartmentalize hyphae but are typically perforated, thereby permitting passage and communication of cytoplasm or even protoplasm between compartments. However, septal pores can become blocked by Woronin bodies or other materials. This aids morphological and biochemical differentiation and serves to seal off stressed or damaged hyphae from undamaged colony regions. Again, different pore types are representative of different taxonomic groups and species (Table 1.2).

In yeasts, the cell wall structure comprises polysaccharides (predominantly β-glucans for rigidity) and proteins (mainly mannoproteins on the outermost layer for determining porosity), together with some lipid and inorganic phosphate material. Hyphal cell walls generally contain fewer mannans than yeast cell forms, and such changes in composition are even observed during the transition from unicellular to mycelial growth of dimorphic fungi. Figure 1.3 shows the composition and structure of the *S. cerevisiae* cell wall.

Chitin is also found in yeast cell walls and is a major constituent of bud scars (see Figure 1.1). These are remnants of previous budding events found on the surface of mother cells following birth of daughter cells (buds). The chitin-rich

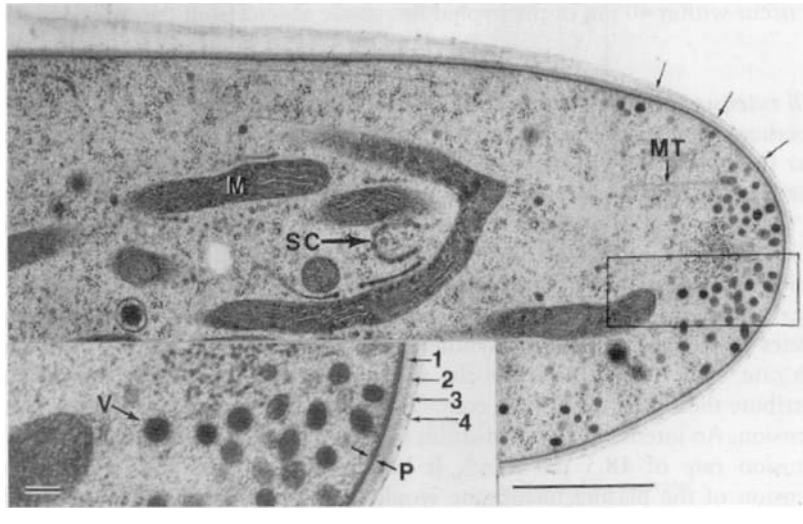

Figure 1.2 Transmission electron microscopy of ultrathin sections of fungal cells reveals intracellular fine structure – M, mitochondrion; V, vacuole (from Carlile, Watkinson and Gooday, 2001)

bud scars of yeast cells can be stained with fluorescent dyes (e.g. calcoflour white) and this can provide useful information regarding cellular age, since the number of scars represents the number of completed cell division cycles. Outside the cell wall in fungi, several extramural layers may exist including fimbriae and capsules. Fungal fimbriae are long, protein-containing protrusions appearing from the cell wall of certain basidiomycetous and ascomycetous fungi that are involved in cell–cell conjugation. Capsules are extracellular polysaccharide-containing structures found in basidiomycetous fungi that are involved in stress protection. In the opportunistically pathogenic yeast, *Cryptococcus neoformans*, the capsule may determine virulence properties and evasion from macrophages. One extrahyphal substance, the polymer pullulan, is produced commercially from *Aureobasidium pullulans*.

1.3.2 Subcellular architecture and organelle function

Transmission electron microscopy of ultrathin sections of fungal cells reveals intracellular fine structure (see Figures 1.2 and 1.4). Subcellular compartments (organelles) are bathed in an aqueous cytoplasm containing soluble proteins and other macromolecules together with low-molecular-weight metabolites, although the hyphae of central and therefore older colony regions of filamentous fungi may become devoid of protoplasm, as it is driven forward with the growing tip. Cytoplasmic components additionally comprise microbodies, ribosomes, proteasomes, lipid particles and a cytoskeletal network. The latter confers structural stability to the fungal cytoplasm and consists of microtubules and microfilaments. The following membrane-bound organelles may be found in a typical fungal cell: nucleus, endoplasmic reticulum (ER), mitochondria, Golgi apparatus, secretory vesicles and vacuoles. Several of these organelles form extended membranous systems. For example, the ER is contiguous with the nuclear membrane and secretion of fungal proteins involves inter-membrane trafficking in which the ER, Golgi apparatus, plasma membrane and vesicles all participate.

The nucleus is the structure that defines the eukaryotic nature of fungal cells. It is double membraned and encases the chromosomes in a nucleoplasm. Most yeast and fungi have mainly haploid life cycles, although some (e.g. *S. cerevisiae*) may alternate between haploidy and diploidy. Chromosomes comprise DNA–protein structures that replicate and segregate to newly divided cells or hyphal compartments at mitosis. This, of course, ensures that genetic material is passed on to daughter cells or septated compartments at cell division. Yeasts usually contain a single nucleus per cell. However, the hyphal compartments of filamentous fungi may contain one or more nuclei. Monokaryotic basidiomycetes possess one type of nucleus per compartment whereas dikaryons or heterokaryons possess two or more genetically distinct haploid nuclei. The maintenance of multiple nuclei within individual hyphal compartments allows

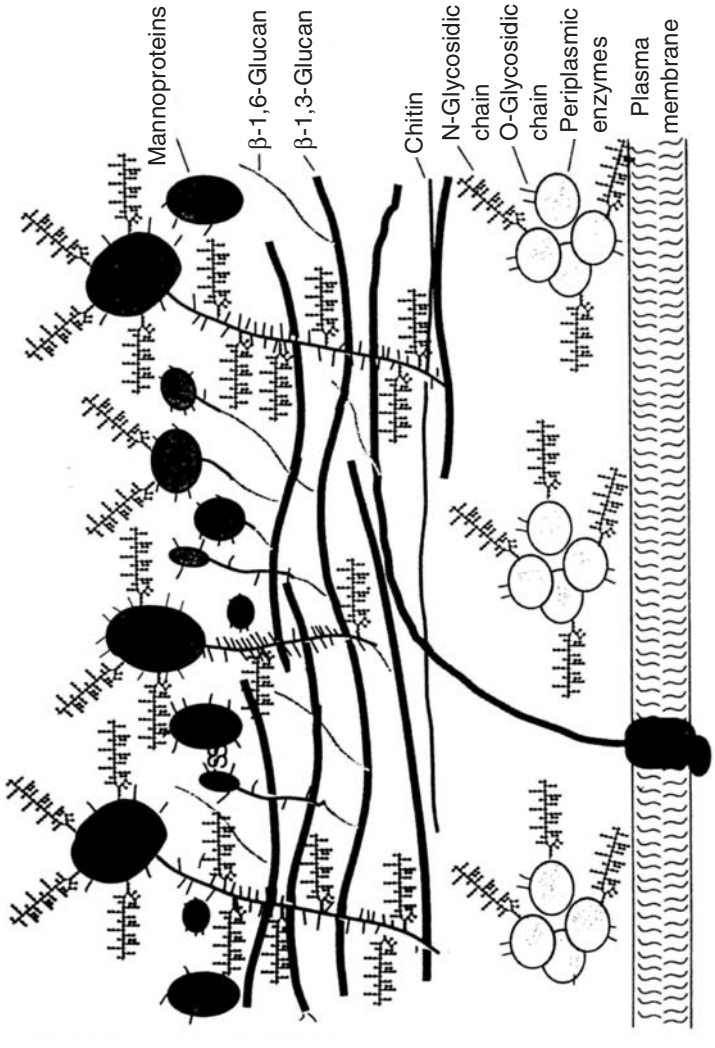

Figure 1.3 Cell envelope structure of the yeast *S. cerevisiae* (from Walker, 1998)

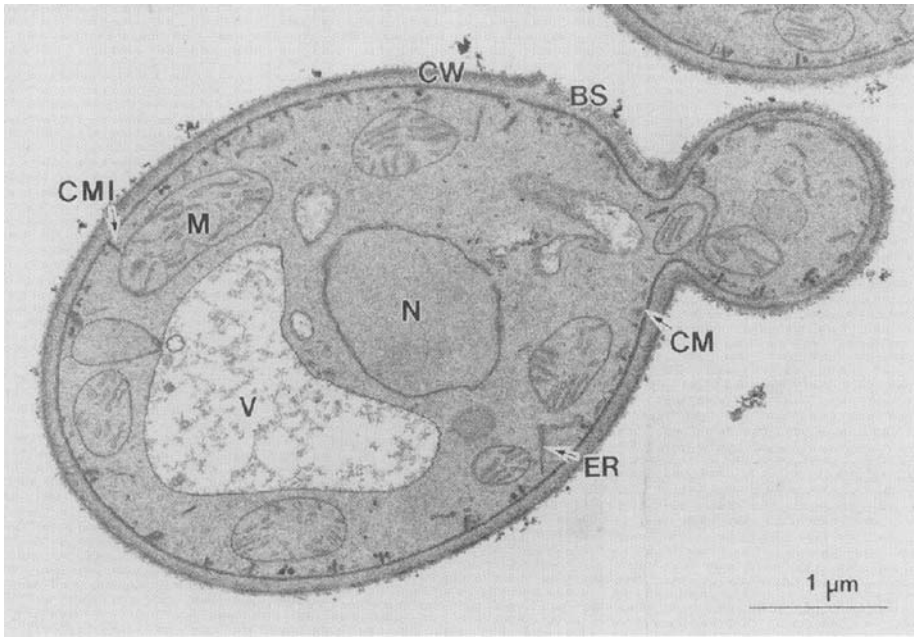

Figure 1.4 Electron micrograph of a typical budding yeast cell – CW, cell wall; CM, cell membrane; CMI, cell membrane invagination; BS, bud scar; M, mitochondrion; N, nucleus; V, vacuole; ER, endoplasmic reticulum (Reproduced with kind permission of Professor Masako Osumi, Japan Women's University, Tokyo)

fungi to take advantage of both haploid and diploid lifestyles. This is discussed further in Chapter 2. The physiological function of the various fungal cell organelles is summarized in Table 1.3.

In filamentous fungi, a phase-dark near-spherical region, which also stains with iron-haemotoxylin, is evident by light microscopy at the apex during hyphal tip growth. The region is termed the Spitzenkörper, the apical vesicle cluster or centre or apical body, as it consists of masses of small membrane-bound vesicles around a vesicle-free core with emergent microfilaments and microtubules. The Spitzenkörper contains differently sized vesicles derived from Golgi bodies, either large vesicles or microvesicles (chitosomes), with varying content. It orientates to the side as the direction of tip growth changes, and disappears when growth ceases. This vesicle supply centre is involved in wall extension and hence tip growth, branching, clamp connection formation and germ tube formation.

1.4 Fungal nutrition and cellular biosyntheses

1.4.1 Chemical requirements for growth

Yeasts and fungi have relatively simple nutritional needs and most species would be able to survive quite well in aerobic conditions if supplied with glucose,

Table 1.3 Functional components of an idealized fungal cell

Organelle or cellular structure	Function
Cell envelope	Comprising the plasma membrane which acts as a selectively permeable barrier for transport of hydrophilic molecules in and out of fungal cells; the periplasm containing proteins and enzymes unable to permeate the cell wall; the cell wall, which provides protection and shape and is involved in cell–cell interactions, signal reception and specialized enzyme activities; fimbriae involved in sexual conjugation; capsules to protect cells from dehydration and immune cell attack
Nucleus	Relatively small. Containing chromosomes (DNA–protein complexes), which pass genetic information to daughter cells at cell division, and the nucleolus, which is the site of ribosomal RNA transcription and processing
Mitochondria	Site of respiratory metabolism under aerobic conditions and, under anaerobic conditions, for fatty acid, sterol and amino-acid metabolism
Endoplasmic reticulum	Ribosomes on the rough ER are the sites of protein biosynthesis
Proteasome	Multi-subunit protease complexes involved in regulating protein turnover
Golgi apparatus and vesicles	Secretory system for import (endocytosis) and export (exocytosis) of proteins
Vacuole	Intracellular reservoir (amino acids, polyphosphate, metal ions); proteolysis; protein trafficking; control of cellular pH. In filamentous fungi, tubular vacuoles transport materials bi-directionally along hyphae
Peroxisome	Oxidative utilization of specific carbon and nitrogen sources (contain catalase, oxidases). Glyoxysomes contain enzymes of the glyoxylate cycle

ammonium salts, inorganic ions and a few growth factors. Macronutrients, supplied at millimolar concentrations, comprise sources of carbon, nitrogen, oxygen, sulphur, phosphorus, potassium and magnesium; and micronutrients, supplied at micromolar concentrations, comprise trace elements such as calcium, copper, iron, manganese and zinc that would be required for fungal cell growth (see Table 1.4). Some fungi are oligotrophic, apparently growing with very limited nutrient supply, surviving by scavenging minute quantities of volatile organic compounds from the atmosphere.

Table 1.4 Elemental requirements of fungal cells

Element	Common sources	Cellular functions
Carbon	Sugars	Structural element of fungal cells in combination with hydrogen, oxygen and nitrogen. Energy source
Hydrogen	Protons from acidic environments	Transmembrane proton motive force vital for fungal nutrition. Intracellular acidic pH (around 5–6) necessary for fungal metabolism
Oxygen	Air, O_2	Substrate for respiratory and other mixed-function oxidative enzymes. Essential for ergosterol and unsaturated fatty acid synthesis
Nitrogen	NH_4^+ salts, urea, amino acids	Structurally and functionally as organic amino nitrogen in proteins and enzymes
Phosphorus	Phosphates	Energy transduction, nucleic acid and membrane structure
Potassium	K^+ salts	Ionic balance, enzyme activity
Magnesium	Mg^{2+} salts	Enzyme activity, cell and organelle structure
Sulphur	Sulphates, methionine	Sulphydryl amino acids and vitamins
Calcium	Ca^{2+} salts	Possible second messenger in signal transduction
Copper	Cupric salts	Redox pigments
Iron	Ferric salts: Fe^{3+} is chelated by siderophores and released as Fe^{2+} within the cell	Haem proteins, cytochromes
Manganese	Mn^{2+} salts	Enzyme activity
Zinc	Zn^{2+} salts	Enzyme activity
Nickel	Ni^{2+} salts	Urease activity
Molybdenum	Na_2MoO_4	Nitrate metabolism, vitamin B_{12}

Being chemo-organotrophs, fungi need fixed forms of organic compounds for their carbon and energy supply. Sugars are widely utilized for fungal growth, and can range from simple hexoses such as glucose to polysaccharides such as starch, cellulose and aromatic hydrocarbons (inc. lignin). Table 1.5 outlines the variety of carbon sources that can be utilized by yeasts and filamentous fungi for growth.

Fungi are non-diazotrophic (cannot fix nitrogen) and need to be supplied with nitrogen-containing compounds, either in inorganic form such as ammonium salts, or in organic form such as amino acids. Ammonium sulphate is a commonly used nitrogen source in fungal growth media since it also provides a source of utilizable sulphur. Many fungi (but not the yeast *S. cerevisiae*) can also grow on nitrate, and if able to do so may also utilize nitrite. Nitrate reductase, followed by nitrite reductase, are the enzymes responsible for converting nitrate to ammonia. Most fungi can assimilate amino acids, amines and amides as nitrogen sources. Urea utilization is common in fungi and some basidiomycetous yeasts are classed as urease positive (able to utilize urea) whilst most ascomycetous yeasts are urease negative.

In terms of oxygen requirements, most fungi are aerobes. Although yeasts such as *S. cerevisiae* are sometimes referred to as facultative anaerobes, they cannot actually grow in strictly anaerobic conditions unless supplied with certain fatty acids and sterols (which they cannot synthesize without molecular oxygen). For aerobically respiring yeasts and fungi, oxygen is required as the terminal electron acceptor. Different fungal species respond to oxygen availability in diverse ways and Table 1.6. categorizes fungi into different groups on this basis.

Sulphur sources for fungal growth include sulphate, sulphite, thiosulphate, methionine and glutathione with inorganic sulphate and the sulphur amino acid methionine being effectively utilized. Virtually all yeasts can synthesize sulphur amino acids from sulphate, the most oxidized form of inorganic sulphur.

Phosphorus is essential for biosynthesis of fungal nucleic acids, phospholipids, ATP and glycophosphates. Hence, the phosphate content of fungi is considerable (e.g. in yeast cells it accounts for around three to five per cent of dry weight; the major part of this is in the form of orthophosphate ($H_2PO_4^-$), which acts as a substrate and enzyme effector). The fungal vacuole can serve as a storage site for phosphate in the form of polyphosphates. Both nitrogen and phosphorus availability may be growth limiting in nature. Filamentous fungi have evolved a number of biochemical and morphological strategies allowing capture of often poorly available phosphorus within the natural environment. Plants exploit such efficiency during symbioses between their roots and certain mycorrhizal fungi.

Concerning requirements for minerals, potassium, magnesium and several trace elements are necessary for fungal growth. K and Mg are macroelements required in millimolar concentrations whereas other microelements (trace elements) are generally required in the micromolar range. These include Mn, Ca

Table 1.5 Diversity of carbon sources for yeast and filamentous fungal growth (adapted from Walker, 1998)

Carbon source	Typical examples	Comments
Hexose sugars	D-glucose, D-galactose	Glucose metabolized by majority of yeasts and filamentous fungi
	D-fructose, D-mannose	If a yeast does not ferment glucose, it will not ferment other sugars. If a yeast ferments glucose, it will also ferment fructose and mannose, but not necessarily galactose
Pentose sugars	L-arabinose, D-xylose, D-xyulose, L-rhamnose	Some fungi respire pentoses better than glucose. *S. cerevisiae* can utilize xyulose but not xylose
Disaccharides	Maltose, sucrose, lactose, trehalose, melibiose, cellobiose, melezitose	If a yeast ferments maltose, it does not generally ferment lactose and vice versa. Melibiose utilization used to distinguish ale and lager brewing yeasts. Large number of yeasts utilize disaccharides. Few filamentous fungi (e.g. *Rhizopus nigricans*) cannot utilize sucrose
Trisaccharides	Raffinose, maltotriose	Raffinose only partially used by *S. cerevisiae*, but completely used by other *Saccharomyces* spp. (*S. carlsbergensis*, *S. kluyveri*)
Oligosaccharides	Maltotetraose, maltodextrins	Metabolized by amylolytic yeasts, not by brewing strains
Polysaccharides	Starch, inulin, cellulose, hemicellulose, chitin, pectic substances	Polysaccharide-fermenting yeasts are rare. *Saccharomycopsis* spp. and *S. diastaticus* can utilize soluble starch; *Kluyveromyces* spp. possess inulinase. Many filamentous fungi can utilize these depending on extracellular enzyme activity
Lower aliphatic alcohols	Methanol, ethanol	Respiratory substrates for many fungi. Several methylotrophic yeasts have industrial potential
Sugar alcohols	Glycerol, glucitol	Can be respired by yeasts and a few fungi
Organic acids	Acetate, citrate, lactate, malate, pyruvate, succinate	Many yeasts can respire organic acids, but few can ferment them
Fatty acids	Oleate, palmitate	Several species of oleaginous yeasts can assimilate fatty acids as carbon and energy sources

Table 1.5 *Continued*

Carbon source	Typical examples	Comments
Hydrocarbons	n-alkanes	Many yeast and a few filamentous species grown well on C_{12}–C_{18} n-alkanes
Aromatics	Phenol, cresol, quinol, resourcinol, catechol, benzoate	Few yeasts can utilize these compounds. Several n-alkane-utilizing yeasts use phenol as carbon source via the β-ketoadipate pathway
Miscellaneous	Adenine, uric acid, butylamine, pentylamine, putrescine	Some mycelial fungi and yeasts, for example *Arxula adeninivorans* and *A. terestre*, can utilize such compounds as sole source of carbon and nitrogen
	Lignin	Can be decayed only by white-rot fungi (basidiomycotina). Little net energy gained directly, but makes available other polysaccharides such as cellulose and hemicellulose
	'Hard' keratin	Keratinophilic fungi

Fe, Zn, Cu, Ni, Co and Mo. Table 1.7 summarizes the main metals required for fungal growth. Toxic minerals (e.g. Ag, As, Ba, Cs, Cd, Hg, Li, Pb) adversely affect fungal growth at concentrations greater than 100 μM.

Fungal growth factors are organic compounds occasionally needed in very low concentrations for specific enzymatic or structural roles, but not as energy sources. These include vitamins (e.g. thiamin, biotin), purines, pyrimidines, nucleosides, nucleotides, amino acids, fatty acids and sterols. For fungi to have a growth factor requirement indicates that cells cannot synthesize the particular factor, resulting in the curtailment of growth without its provision in culture media. Some fungi (e.g. *Aspergillus niger*, *Penicillium chrysogenum*) have very simple nutritional needs and are able to synthesize their own growth factors from glucose.

1.4.2 Fungal cultivation media

Fungal nutritional requirements are important not only for successful cultivation in the laboratory but also for the optimization of industrial fermentation processes. In the laboratory, it is relatively easy to grow yeasts and fungi on

Table 1.6 Yeast and fungal metabolism based on responses to oxygen availability

Mode of energy metabolism	Examples	Comments
Obligate fermentative	Yeasts: *Candida pintolopesii* (*Saccharomyces telluris*)	Naturally occurring respiratory-deficient yeasts. Only ferment, even in presence of oxygen
	Fungi: facultative and obligate anaerobes	No oxygen requirement for these fungi. Two categories exist with respect to the effects of air: facultative anaerobes (e.g. *Aqualinderella* and *Blastocladia*) and obligate anaerobes (e.g. *Neocallimastix*)
Facultatively fermentative		
Crabtree positive	*Saccharomyces cerevisiae*	Such yeasts predominantly ferment high-sugar-containing media in the presence of oxygen
Crabtree negative	*Candida utilis*	Such yeasts do not form ethanol under aerobic conditions and cannot grow anaerobically
Non-fermentative	Yeasts: *Rhodotorula rubra*	Such yeasts do not produce ethanol, either in the presence or absence of oxygen
	Fungi: *Phycomyces*	Oxygen essential for such (obligately oxidative) fungi
Obligate aerobes	*Gaemannomyces graminis* (the take-all fungus)	The growth of these is markedly reduced if oxygen partial pressure falls below normal atmospheric

Adapted from Walker (1998), Deacon (2000) and Carlile, Watkinson and Gooday (2001).

complex culture media such as malt extract or potato-dextrose agar or broth, which are both carbon rich and in the acidic pH range. Mushrooms are cultivated on various substrates depending in the main on provincial availability. Therefore, *Agaricus bisporus* is grown in the UK, US and France on wheat-straw,

Table 1.7 Metals required for fungal growth and metabolic functions (adapted from Walker, 2004)

Metal ion	Concentration supplied in growth medium*	Main cellular functions
Macroelements		
K	2–4 mM	Osmoregulation, enzyme activity
Mg	2–4 mM	Enzyme activity, cell division
Microelements		
Mn	2–4 μM	Enzyme cofactor
Ca	<1 μM	Second messenger, yeast flocculation
Cu	1.5 μM	Redox pigments
Fe	1–3 μM	Haem proteins, cytochromes
Zn	4–8 μM	Enzyme activity, protein structure
Ni	~10 μM	Urease activity
Mo	1.5 μM	Nitrate metabolism, vitamin B_{12}
Co	0.1 μM	Cobalamin, coenzymes

* Figures relate to yeast (*S. cerevisiae*) growth stimulation, and are dependent on the species/strain and conditions of growth, but they would be generally applicable for fungal growth.

the padi-straw mushroom *Volvariella volvacea* is grown in South-East Asia on damp rice-straw and in Hong Kong on cotton waste, and in Japan the shiitake mushroom *Lentinus edodes* is cultivated on fresh oak logs. In industry, media for fungal fermentation purposes needs to be optimized with regard to the specific application and production process. For some industrial processes, growth media may already be complete, such as malt wort or molasses for brewing or baker's yeast production, respectively (see Table 1.8). However, for other processes, supplementation of agriculturally derived substrates such as corn steep liquor, molasses or malt broth with additional nutrients and growth factors may be necessary. For example, for penicillin production by *Penicillium* spp. the following may constitute a suitable fermentation medium: sucrose (3 g/L), corn steep liquor (100 g/L), KH_2PO_4 (1 g/L), $(NH_4)_2SO_4$ (12 g/L), $CaCl_2.2H_2O$ (0.06 g/L), phenoxyacetic acid (5.7 g/L). (Information from Jorgensen *et al.* 1995 *Biotech. Bioeng.* **46**: 558–572.) Other industrial processes such as the growth of *Fusarium venenatum* for production of Quorn™ mycoprotein require culture on a completely defined medium.

1.4.3 Nutrient uptake and assimilation

Fungal cells utilize a diverse range of nutrients and employ equally diverse nutrient acquisition strategies. Fungi are non-motile, saprophytic (and sometimes parasitic), chemo-organotrophic organisms. Fungi exhibit dynamic interactions

Table 1.8 Principal ingredients of selected industrial media for yeasts and fungi

Components	Molasses	Malt wort	Wine must	Cheese whey	Corn steep liquor
Carbon sources	Sucrose Fructose Glucose Raffinose	Maltose Sucrose Fructose Glucose Maltotriose	Glucose Fructose Sucrose (trace)	Lactose	Glucose, other sugars
Nitrogen sources	Nitrogen compounds as unassimilable nitrogen proteins. Nitrogen sources need to be supplemented	Low-molecular- weight α-amino nitrogen, compounds, ammonium ions and a range of amino acids	Variable levels of ammonia mitrogen, which may be limiting. Range of amino acids	Unassimilable globulin and globulin proteins. Low levels of ammonium and urea nitrogen.	Amino acids, protein
Minerals	Supply of P, K, and S available. High K^+ levels may be inhibitory	Supply of P, K, Mg and and S available	Supply of P, K, Mg and S available. High levels of sulphite often present	Supply of P, K, Mg, S	Supply of P, K, Mg, S.
Vitamins	Small, but generally adequate supplies. Biotin is deficient in beet molasses	Supply of vitamins is usually adequate. High-adjuct-sugar wort may be deficient in biotin	Vitamin supply generally sufficient	Wide range of vitamins present	Biotin, pyridoxine, thiamin
Trace elements	Range of trace metals present, although Mn^{2+} may be limiting	All supplied, although Zn^{2+} may be limiting	Sufficient quantitities available. Unfermentable	Fe, Zn, Mn, Ca, Cu present	Range of trace elements present
Other components	Unfermentable sugars (2–4%), organic acids, waxes, pigments, silica, pesticide residues, caramelized compounds, betaine	Unfermentable maltodextrins, pyrazines, hop compounds	Pentoses. Tartaric and malic acids. Decanoic and octanoic acids may be inhibitory. May be deficient in sterols and unsaturated fatty acids	Lipids, NaCl. Lactic and citric acids	High levels of lactic acid present. Fat and fibre also present

with their nutritional environment that may be exemplified by certain morphological changes depending on nutrient availability. For example, the filamentous mode of growth observed at the periphery of yeast colonies growing in agar is akin to a foraging for nutrients as observed in certain eucarpic fungi. Metabolic dynamism is also evident in yeasts, which, although not avid secretors of hydrolytic enzymes like higher filamentous fungi, are nevertheless able to secrete enzymes to degrade polymers such as starch (amylolytic yeasts such as *Schwanniomyces occidentalis*).

Several cellular envelope barriers to nutrient uptake by fungal cells exist, namely the capsule, the cell wall, the periplasm and the cell membrane. Although not considered as a freely porous structures, fungal cell walls are relatively porous to molecules up to an average molecular mass of around 300 Da, and will generally retain molecules greater than around 700 Da. Generally, fungi can absorb only small soluble nutrients (monosaccharides, amino acids or small peptides).

The plasma membrane is the major selectively permeable barrier that dictates nutrient entry and metabolite exit from the fungal cell. Membrane transport mechanisms are important in fungal physiology since they govern the rates at which cells metabolize, grow and divide. Fungi possess different modes of passive and active uptake at the plasma membrane: free diffusion, facilitated diffusion, diffusion channels and active transport (as summarized in Table 1.9). Active transport of nutrients such as sugars, amino acids, nitrate, ammonium, sulphate and phosphate in filamentous fungi involves spatial separation of the ion pumps mostly behind the apex, whereas the symport proteins are active close to the tip. Thus, nutrient uptake occurs at the hyphal tip as it continuously drives into fresh resource, and the mitochondria localized behind the apex supply ATP to support the ion pump.

1.4.4 Overview of fungal biosynthetic pathways

Anabolic pathways are energy consuming, reductive processes that lead to the biosynthesis of new cellular material and are mediated by dehydrogenase enzymes, which predominantly use reduced NADP as redox cofactors. NADPH is generated by the hexose monophosphate pathway (or Warburg–Dickens pathway), which accompanies glycolysis (see Section 1.5 below). In *S. cerevisiae*, up to 20 per cent of total glucose may be degraded via the hexose monophosphate pathway. This pathway generates cytosolic NADPH (following the dehydrogenation of glucose 6-phosphate using glucose 6-phosphate dehydrogenase and NADP as hydrogen acceptor) for biosynthetic reactions leading to the production of fatty acids, amino acids and sugar alcohols, structural and storage polysaccharides and secondary metabolites. Besides generating NADPH, the hexose monophosphate pathway also produces ribose sugars for the synthesis of nucleic acids, RNA and DNA, and for nucleotide coenzymes, NAD, NADP, FAD and FMN. This is summarized as follows:

Table 1.9 Modes of nutrient transport in fungi

Mode of nutrient transport	Description	Examples of nutrients transported
Free diffusion	Passive penetration of lipid-soluble solutes through the plasma membrane following the law of mass action from a high extracellular concentration to a lower intracellular concentration	Organic acids, short-chain alkanes and long-chain fatty acids by fungi and the export of lipophilic metabolites (e.g. ethanol) and gaseous compounds
Facilitated diffusion	Translocates solutes down a transmembrane concentration gradient in an enzyme- (permease-) mediated manner. As with passive diffusion, nutrient translocation continues until the intracellular concentrations equal to that of the extracellular medium	In the yeast *S. cerevisiae*, glucose is transported in this manner
Diffusion channels	These operate as voltage-dependent 'gates' to transiently move certain nutrient ions down concentration gradients. They are normally closed at the negative membrane potential of resting yeast cells but open when the membrane potential becomes positive	Ions such as potassium may be transported in this fashion
Active transport	The driving force is the membrane potential and the transmembrane electrochemical proton gradient generated by the plasma membrane H^+-ATPase. The latter extrudes protons using the free energy of ATP hydrolysis that enables nutrients to either enter with influxed protons, as in 'symport' mechanisms, or against effluxed protons, as in 'antiport' mechanisms	Many nutrients (sugars, amino acids, ions)

$$\text{glucose 6-phosphate} + 2\text{NADP}^+ \rightarrow \text{ribulose 5-phosphate} + \text{CO}_2 + \text{NADPH} + 2\text{H}^+$$

and complete oxidation of glucose 6-phosphate would result in

$$\text{glucose 6-phosphate} + 12\text{NADP}^+ \rightarrow 6\text{CO}_2 + 12\text{NADPH} + 12\text{H}^+ + \text{P}_i$$

Fungal growth on non-carbohydrate substrates as sole carbon sources (e.g. ethanol, glycerol, succinate and acetate) may lead to gluconeogenesis (conversion of pyruvate to glucose) and polysaccharide biosynthesis. Gluconeogenesis may be regarded as a reversal of glycolysis and requires ATP as energy and NADH as reducing power.

Concerning fungal amino-acid biosynthesis, simple nitrogenous compounds such as ammonium may be assimilated into amino-acid *families,* the carbon skeletons of which originate from common precursors of intermediary carbon metabolism.

The two main fungal storage carbohydrates are glycogen and trehalose. Glycogen is similar to starch with β-1,4-glucan linear components and β-1,6-branches. Trehalose is a disaccharide of glucose. Both trehalose and glycogen are synthesized following the formation of UDP-glucose, catalysed by UDP-glucose pyrophosphorylase:

$$\text{UTP} + \text{glucose 1-phosphate} \rightarrow \text{UDP-glucose} + \text{pyrophosphate}$$

Glycogen is synthesized by glycogen synthase. Glycogen may be metabolized by glycogen phosphorylase when nutrients become limited under starvation conditions and this contributes to the maintenance metabolism of cells by furnishing energy in the form of ATP. In yeast cells, glycogen breakdown is accompanied by membrane sterol biosynthesis and this is important for brewing yeast vitality and successful fermentation. The other major storage carbohydrate, trehalose, is synthesized from glucose 6-phosphate and UDP-glucose by trehalose 6-phosphate synthase and converted to trehalose by a phosphatase. In addition to a storage role, trehalose is an important translocation material in filamentous forms and is also involved in stress protection in yeasts and fungi, accumulating when cells are subject to environmental insults such as heat shock or osmostress, or during plant host–fungal parasite interactions. Polyols such as mannitol derived from fructose phosphate, are also translocated.

1.4.5 Fungal cell wall growth

The structural polysaccharides in fungal cell walls include mannans, glucans and chitin and are synthesized from sugar nucleotide substrates formed by pyrophosphorylase enzymes. For example:

$$\text{glucose 1-phosphate} + \text{UTP} \rightarrow \text{UDF-glucose} + \text{PP}_\text{i}$$
$$\text{mannose 1-phosphate} + \text{GTP} \rightarrow \text{GDP-mannose} + \text{PP}_\text{i}$$

Glucan synthesis involves plasma-membrane-associated glucan synthetases for assembly of β-1,3 linkages and β-1,6 branches of cell wall glucan. Chitin (a polymer of N-acetylglucosamine) is an important fungal cell wall structural

component and is involved in the yeast budding process and in dimorphic transitions from yeast to filamentous forms. Chitin synthetases catalyse the transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to a growing chitin polymer within the cell wall. Mannoproteins pre-assembled within the Golgi are delivered to the cell wall via vesicles from the vesicle supply centre. Various vesicles, containing wall-synthetic enzymes, wall-lytic enzymes, enzyme activators and certain pre-formed wall components, are transported to the growing hyphal tip, where they fuse with the plasma membrane and release their contents, which together with substrates delivered from the cytosol synthesize the growing cell wall.

1.5 Fungal metabolism

1.5.1 Carbon catabolism

Being chemo-organotrophs, fungi derive their energy from the breakdown of organic compounds. Generally speaking, fungi, but few yeast species, extracellularly break down polymeric compounds by secreted enzymes prior to utilization of monomers as carbon and energy sources. Due to their relatively large size (20–60 kDa), enzymes assembled by the Golgi are transported in vesicles to be secreted from sites of cell growth, essentially from extending hyphal tips. Enzymes may either become linked to the cell wall as wall-bound enzymes or may diffuse externally to decay substrates within the local environment.

Some examples follow of hydrolytic, oxidative, peroxidative and free-radical-generating enzyme systems produced by fungi for the degradation of polymeric compounds:

$$\text{pectin} \xrightarrow{\text{pectin lyase, polygalactorunase}} \text{galacturonic acid}$$

$$\text{starch} \xrightarrow{\text{amylases, glucoamylase}} \text{glucose}$$

$$\text{inulin} \xrightarrow{\text{inulinase}} \text{fructose}$$

$$\text{cellulose} \xrightarrow{\text{cellulases}} \text{glucose}$$

$$\text{hemicellulose} \xrightarrow{\text{hemicellulases, xylanase}} \text{xylose, glucose}$$

$$\text{lipids} \xrightarrow{\text{lipases}} \text{fatty acids}$$

$$\text{proteins} \xrightarrow{\text{proteinases}} \text{amino acids}$$

$$\text{chitin} \xrightarrow{\text{chitinase}} \text{N-acetylglucosamine}$$

$$\text{lingnin} \xrightarrow{\text{ligninase, manganese peroxidase, laccase, glucose oxidase}} \text{variety of largely phenolic products}$$

Several lipolytic yeasts are known (e.g. *Candida rugosa*, *Yarrowia lipolytica*) that secrete lipases to degrade triacylgycerol substrates to fatty acids and

glycerol. In wood, the cellulose and hemicellulose components are embedded within a heteropolymeric 3D lignin matrix, thus forming a complex lignocellulose material. Only certain filamentous basidiomycete or ascomycete fungi are able to degrade the recalcitrant lignin component, making available the cellulose or hemicellulose components. These are known as white-rot fungi due to resultant colouration of the delignified wood. Such fungi employ a cocktail of oxidative (including laccase) and peroxidative enzymes, together with hydrogen-peroxide-generating enzyme systems, to attack the at least 15 different inter-unit bond types extant within the lignin polymer. The manganese and lignin peroxidase enzyme systems operate by releasing highly reactive but transient oxygen free radicals, which bombard and react with parts of the lignin molecule, generating a chain of chemical oxidations and producing a range of mainly phenolic end products. White-rot fungi have applications in, for example, upgrading lignocellulose waste for animal feed, paper production and bleaching and the bioremediation of contaminated land and water. Brown-rot and soft-rot (in wet wood) fungi are only able to degrade the cellulose and hemicellulose components of wood. Cellulose decomposition involves the synergistic activity of endoglucanases (hydrolyse the internal bonds of cellulose), exoglucanases (cleave cellobiose units from the end of the cellulose chain) and glucosidase (hydrolyse cellobiose to glucose). Initial attack of cellulose microfibrills within the cell wall may involve the generation of hydrogen peroxide.

The sequence of enzyme-catalysed reactions that convert glucose to pyruvic acid is known as glycolysis, and this pathway provides fungal cells with energy, together with precursor molecules and reducing power (in the form of NADH) for biosynthetic pathways. In serving both catabolic and anabolic functions, glycolysis is sometimes referred to as being an amphibolic pathway. Glycolysis may be summarized as follows:

$$\text{glucose} \rightarrow 2\text{pyruvate} + 2\text{ATP} + 2\text{NADH} + \text{H}^+$$

During glycolysis, glucose is phosphorylated using ATP to eventually produce fructose 1,6-biphosphate, which is then split by aldolase to form two triose phosphate compounds. Further phosphorylation occurs, forming two triose diphosphates, from which four H atoms are accepted by two molecules of NAD. In the latter stages of glycolysis, four molecules of ATP are formed (by transfer of phosphate from the triose diphosphates to ADP), and this results in the formation of two molecules of pyruvic acid. ATP production during glycolysis is referred to as substrate-level phosphorylation.

In yeast cells undergoing alcoholic fermentation of sugars under anaerobic conditions, NAD is regenerated in terminal step reactions from pyruvate. In the first of these, pyruvate is decarboxylated (by pyruvate decarboxylase) before a final reduction, catalysed by alcohol dehydrogenase (ADH), to ethanol. Such

regeneration of NAD prevents glycolysis from stalling and maintains the cell's oxidation–reduction balance. Additional minor fermentation metabolites are produced by fermenting yeast cells, including glycerol, fusel alcohols (e.g. isoamyl alcohol), esters, (e.g. ethyl acetate), organic acids (e.g. citrate, succinate, acetate) and aldehydes (e.g. acetaldehyde). Such compounds are important in flavour development in alcoholic beverages such as beer, wine and whisky.

Aerobic dissimilation of glucose by fungi leads to respiration, which is the major energy-yielding metabolic route and involves glycolysis, the citric acid cycle, the electron transport chain and oxidative phosphorylation. In addition to glucose, many carbon substrates can be respired by fungi, including pentose sugars (e.g. xylose), sugar alcohols (e.g. glycerol), organic acids (e.g. acetic acid), aliphatic alcohols (e.g. methanol, ethanol), hydrocarbons (e.g. n-alkanes) and aromatic compounds (e.g. phenol). Fatty acids are made available for fungal catabolism following extracellular lipolysis of fats and are metabolized by β-oxidation in mitochondria.

During glucose respiration under aerobic conditions, pyruvate enters the mitochondria, where it is oxidatively decarboxylated to acetyl CoA by pyruvate dehydrogenase, which acts as the link between glycolysis and the cyclic series of enzyme-catalysed reactions known as the citric acid cycle (or Kreb's cycle). This cycle represents the common pathway for the oxidation of sugars and other carbon sources in yeasts and filamentous fungi and results in the complete oxidation of one pyruvate molecule to $2CO_2$, 3NADH, $1FADH_2$, $4H^+$ and 1GTP. Like glycolysis, the citric acid cycle is amphibolic since it performs both catabolic and anabolic functions, the latter providing intermediate precursors (e.g. oxaloacetate and α-ketoglutarate) for the biosynthesis of amino acids and nucleotides. The removal of intermediates necessitates their replenishment to ensure continued operation of the citric acid cycle. The glyoxylate cycle is an example of such an *anaplerotic* reaction and involves the actions of the enzymes pyruvate carboxylase,

$$\text{pyruvate} + CO_2 + \text{ATP} + H_2O \rightarrow \text{oxaloacetate} + \text{ADP} + P_i$$

and phosophoenolpyruvate carboxykinase,

$$\text{phosphoenolpyruvate} + CO_2 + H_2O \rightarrow \text{oxaloacetate} + H_3PO_4$$

During the citric acid cycle, dehydrogenase enzymes transfer hydrogen atoms to the redox carriers NAD and FAD, which become reduced. On the inner membrane of mitochondria, these reduced coenzymes are then reoxidized and oxygen is reduced to water via the electron transport chain. Energy released by electron transfer is used to synthesize ATP by a process called oxidative phosphorylation. The chemiosmotic theory describes proton pumping across the inner mitochondrial membrane to create a transmembrane proton gradient (ΔpH) and a membrane potential difference. Together, these comprise the proton motive force

Table 1.10 Respiratory chain characteristics of yeasts and fungi (adapted from Walker, 1998)

Type	Typical species	Sensitive to	Insensitive to
Normal respiration	All aerobic fungi	Cyanide and low azide[1]	SHAM[2]
Classic alternative	*Yarrowia lipolytica* (and in stationary phase cultures of several yeast species)	SHAM	Cyanide, high azide
New alternative	*Schizosaccharomyces pombe* *Saccharomyces cerevisiae* *Kluyveromyces lactis* *Williopsis saturnus*	High azide	Cyanide, low azide, SHAM

[1] The azide-sensitive pathway lacks proton transport capability and accepts electrons from NADH but not from succinate.
[2] SHAM = salicy hydroxamate. The SHAM-sensitive pathway transports electrons to oxygen also without proton transport, and therefore does not phosphorylate ADP.

that is the driving force for ATP synthesis. Each pair of electrons in NADH yields about 2.5 ATP while residual energy is largely dissipated as metabolic heat. Since mitochondria are impermeable to NADH, this reduced coenzyme generated in the cytoplasm during glycolysis is 'shuttled' across the mitochondrial membrane using either the *glycerophosphate shuttle* (which uses NADH to reduce dihydroxyacetone phosphate to glycerol 3-phosphate) or the *malate shuttle* (which uses NADH to reduce oxaloacetate to malate). These processes enable molecules to be oxidized within mitochondria to yield reduced cofactors which in turn are oxidized by the electron transport chain.

Fungi use molecular oxygen as a terminal electron acceptor in aerobic respiration in different ways (see Table 1.10). Some yeasts, including *S. cerevisiae*, exhibit *alternative respiration* characterized by insensitivity to cyanide but sensitivity to azide.

1.5.2 Nitrogen metabolism

Fungi assimilate simple nitrogenous sources for the biosynthesis of amino acids and proteins. For example, ammonium ions are readily translocated and can be directly assimilated into the amino acids glutamate and glutamine, which serve as precursors for the biosynthesis of other amino acids. Proteins can also be utilized following release of extracellular protease enzymes. Glutamate is a key compound in both nitrogen and carbon metabolism and glutamine synthetase is important as it catalyses the first step in pathways leading to the synthesis of many impor-

tant cellular macromolecules. Other important enzymes of fungal nitrogen metabolism include glutamate dehydrogenase and glutamate synthase (glutamine amide: 2-oxoglutarate-aminotransferase, or GOGAT), the latter requiring ATP. When glutamine synthetase is coupled with glutamate synthase this represents a highly efficient 'nitrogen-scavenging' process for fungi to assimilate ammonia into amino acids and citric acid cycle intermediates. The particular route(s) of ammonium assimilation adopted by fungi depend on the concentration of available ammonium ions and the intracellular amino acid pools.

Some yeasts and fungi can use nitrate as a sole source of nitrogen through the activities of nitrate reductase,

$$NO_3^- \longrightarrow NO_2^-$$

and nitrite reductase,

$$NO_2^- \longrightarrow NH_4^+$$

The resulting ammonium ions can then be assimilated into glutamate and glutamine, which represent end products of nitrate assimilation by yeasts.

Urea can also be utilized following its conversion to ammonium by urea aminohydrolase (urea carboxylase plus allophanate hydrolase):

$$NH_2CONH_2 + ATP + HCO_3^- \rightarrow NH_2CONHCOO^- \rightarrow 2NH_4^+ + 2HCO_3^-$$

Amino acids can either be assimilated into proteins or dissimilated by decarboxylation, deamination, transamination and fermentation. Amino-acid degradation by yeasts and fungi yields both ammonium and glutamate. During fermentation, yeasts may produce higher alcohols or *fusel oils* such as isobutanol and isopentanol following amino-acid deamination and decarboxylation. These represent important flavour constituents in fermented beverages.

1.6 Fungal growth and reproduction

1.6.1 Physical requirements for growth

Most yeast and fungal species thrive in warm, sugary, acidic and aerobic conditions. As for temperature, the range for fungal growth is quite wide, but generally speaking most species grow very well around 25 °C. Low-temperature psychrophilic fungi and high-temperature thermophilic fungi do, however, exist in nature. Fungal growth at various temperatures depends not only on the genetic background of the species but also on other prevailing physical growth parameters and nutrient availability. With regard to high-temperature stress on fungal cells, thermal damage can disrupt hydrogen bonding and hydrophobic

interactions, leading to general denaturation of proteins and nucleic acids. Fungi, of course, have no means of regulating their internal temperature, and the higher the temperature the greater the cellular damage, with cell viability declining when temperatures increases beyond growth-optimal levels. Temperature optima vary greatly in fungi, with those termed 'thermotolerant' growing well above 40 °C. Thermotolerance relates to the transient ability of cells subjected to high temperatures to survive subsequent lethal exposures to elevated temperatures, such that *intrinsic* thermotolerance is observed following a sudden heat shock (e.g. to 50 °C), whereas *induced* thermotolerance occurs when cells are pre-conditioned by exposure to a mild heat shock (e.g. 30 minutes at 37 °C) prior to a more severe heat shock. Heat-shock responses in fungi occur when cells are rapidly shifted to elevated temperature and, if this is sub-lethal, induced synthesis of a specific set of proteins, the highly conserved 'heat-shock proteins' (Hsps), occurs. Hsps play numerous physiological roles, including thermoprotection.

High water activity, a_w, is required for growth of most fungi, with a minimum a_w of around 0.65. Water is absolutely essential for fungal metabolism, and any external conditions that result in reduced water availability to cells (i.e. 'water stress') will adversely affect cell physiology. The term water potential refers to the potential energy of water and closely relates to the osmotic pressure of fungal growth media. Species such as *Zygosaccharomyces rouxii*, and some *Aspergillus* species able to grow in low-water-potential conditions (i.e. high sugar or salt concentrations) are referred to as osmotolerant or zerotolerant. *S. cerevisiae* is described as a non-osmotolerant yeast. Mild water stress, or *hypersomotic shock,* occurs in fungi when cells are placed in a medium with low water potential brought about by increasing the solute (e.g. salt, sugar) concentration. Conversely, cells experience a *hypo-osmotic shock* when introduced to a medium of higher osmotic potential (due to reducing the solute concentration). Fungi are generally able to survive such short-term shocks by altering their internal osmotic potential (e.g. by reducing intracellular levels of K^+ or glycerol). Glycerol is an example of a *compatible solute* that is synthesized in order to maintain low cytosolic water activity when the external solute concentration is high. Glycerol can effectively replace cellular water, restore cell volume and enable fungal metabolism to continue. Trehalose, arabitol and mannitol can similarly protect against osmotic stress. Evidence suggests that the accumulation of compatible solutes is attributed not only to their synthesis but also to control of membrane fluidity, thus preventing leakage of these to the external environment.

As for pH, most fungi are acidiophilic and grow well between pH 4 and 6, but many species are able to grow, albeit to a lesser extent, in more acidic or alkaline conditions (around pH 3 or pH 8, respectively). Fungal cultivation media acidified with organic acids (e.g. acetic, lactic acids) are more inhibitory to yeast growth compared with those acidified with mineral acids (e.g. hydrochloric, phosphoric acids) because organic acids can lower intracellular

pH (following their translocation across fungal plasma membranes). This forms the basis of action of weak acid preservatives in inhibiting growth of food spoilage fungi. Many filamentous fungi can alter their local external pH by selective uptake and exchange of ions (NO_3^- or NH_4^+/ H^+), or by excretion of organic acids such as oxalic acid.

Other physical parameters influencing fungal physiology include radiation (light or UV may elicit mycelial differentiation and sporulation in some fungi that produce airborne spores), aeration, pressure, centrifugal force and mechanical shear stress.

1.6.2 Cellular reproduction

Fungal growth involves transport and assimilation of nutrients, followed by their integration into cellular components, followed by biomass increase and eventual cell division. The physiology of vegetative reproduction and its control in fungi has been most widely studied in two model eukaryotes, the budding yeast, *Saccharomyces cerevisiae*, and the fission yeast, *Schizosaccharomyces pombe*.

Budding is the most common mode of vegetative reproduction in yeasts and multilateral budding is typical in ascomycetous yeasts (see Table 1.11). In *S. cerevisiae*, buds are initiated when mother cells attain a critical cell size and this coincides with the onset of DNA synthesis. The budding processes results from localized weakening of the cell wall and this, together with tension exerted by turgor pressure, allows extrusion of cytoplasm in an area bounded by a new cell wall. Cell wall polysaccharides are mainly synthesized by glucan and chitin synthetases. Chitin is a polymer of N-acetylglucosamine and this material forms a ring between the mother cell and the bud that will eventually form the characteristic *bud scar* after cell division.

Fission yeasts, typified by *Schizosaccharomyces* spp., divide exclusively by forming a cell septum, which constricts the cell into two equal-sized daughters. In *Schiz. pombe*, newly divided daughter cells grow in length until mitosis is initiated when cells reach a constant cell length (about 14 μm). The cell septum in *Schiz. pombe* forms by lateral growth of the inner cell wall (the primary septum) and proceeds inwardly followed by deposition of secondary septa. Cellular fission, or transverse cleavage, is completed in a manner resembling the closure of an iris diaphragm.

In certain yeast species, the presence or absence of pseudohyphae and true hyphae can be used as taxonomic criteria (e.g. the ultrastructure of hyphal septa may discriminate between certain ascomycetous yeasts). Some yeasts grow with true hyphae initiated from *germ tubes* (e.g. *Candida albicans*), but others (including *S. cerevisiae*) may grow in a pseudohyphal fashion when starved of nutrients. Filamentous growth of yeasts by hyphal or pseudohyphal extension represents a different developmental pathway that is generally reversible. In

Table 1.11 Modes of vegetative reproduction in yeasts (adapted from Walker, 1998)

Mode	Description	Representative yeast genera
Multilateral budding	Buds may arise at any point on the mother cell surface, but never again at the same site. Branched chaining may occasionally follow multilateral budding when buds fail to separate	*Saccharomyces*, *Zygosaccharomyces*, *Torulaspora*, *Pichia*, *Pachysolen*, *Kluyveromyces*, *Williopsis*, *Debaryomyces*, *Yarrowia*, *Saccharomycopsis*, *Lipomyces*
Bipolar budding	Budding restricted to poles of elongated cells (apiculate or lemon shaped) along their longitudinal axis	*Nadsonia*, *Saccharomycodes* *Haneniaspora*, *Wickerhamia*, *Kloeckera*
Unipolar budding	Budding repeated at same site on mother cell surface	*Pityrosporum*, *Trigonopsis*
Monopolar budding	Buds originate at only one pole of the mother cell	*Malassezia*
Binary fission	A cell septum (cell plate or cross-wall) is laid down within cells after lengthwise growth and which cleaves cells into two	*Schizosaccharomyces*
Bud fission	Broad cross-wall at base of bud forms, which separates bud from mother	Occasionally found in *Saccharomycodes*, *Nadsonia* and *Pityrosporum*
Budding from stalks	Buds formed on short denticles or long stalks	*Sterigmatomyces*
Ballistoconidiogenesis	Ballistoconidia are actively discharged from tapering outgrowths on the cell	*Bullera*, *Sporobolomyces*

other words, cells can revert to yeast unicellular growth in more conducive growth conditions, indicating that a filamentous mode of growth represents an adaptation by yeast to foraging when nutrients are scarce.

What constitutes a cell in filamentous fungi is ambiguous. The apical compartments of higher filamentous fungi are often multinucleate, and so the

process of nuclear replication and segregation into a newly extended septated hyphal compartment is known as the duplication cycle. Thus, *Aspergillus nidulans* apical compartments contain approximately 50 nuclei per compartment produced during a 2 hour duplication cycle period. Continued septation results in the formation of sub-apical compartments containing fewer nuclei. Hyphae also commonly branch, usually at some distance behind the leading growing hyphal tip and often just behind a septum in higher fungi. The processes that control branching are not fully elucidated but branch initiation is associated with the appearance of a Spitzenkörper at the site of tip emergence and extension. Branching allows filamentous fungi to fill space in an efficient and appropriate way, according to local environmental circumstances. So, fungi colonizing nutrient-rich substrata branch frequently, producing dense mycelia for resource exploitation, whereas hyphae colonizing nutrient-poor substrata branch less frequently producing effuse mycelia appropriate for resource exploration.

Rates of branching and tip growth are related to the cytoplasmic volume. Thus, the hyphal growth unit is a measure of the average length of hypha required to support hyphal tip growth. It can be calculated from microscopic preparations growing on agar media as the ratio between the total length of mycelium and the total number of tips. The ratio becomes constant after the initial stages of growth, and is characteristic of each fungal species or strain.

1.6.3 Population growth

When yeast or fungal cells are inoculated into a nutrient medium and incubated under optimal physical growth conditions, a typical batch growth curve will result, comprising lag, exponential and stationary phases. The *lag phase* represents a period of zero population growth and reflects the time required for inoculated cells to adapt to their new physical and chemical growth environment (by synthesizing ribosomes and enzymes). The *exponential phase* is a period of logarithmic cell (or mycelial biomass in the case of filamentous growth) doublings and constant, maximum specific growth rate (μ_{max}, in dimensions of reciprocal time, 1/h), the precise value of which depends on the prevailing growth conditions. If growth is optimal and cells double logarithmically, then

$$\frac{dx}{dt} = \mu_{max} x$$

When integrated, this yields

$$\ln x - \ln x_0 = \mu_{max} t$$

(where x_0 is the initial cell mass) or

$$x = x_o e^{(\mu_{max} t)}$$

which is the fundamental equation for exponential batch growth. According to these kinetic expressions a plot of $\ln x$ versus time is linear with the slope being μ_{max}. Calculation of the doubling time (t_d) of a yeast or fungal culture can be achieved from knowledge of μ_{max} as follows:

$$t_d = \frac{\ln 2}{\mu_{max}} = \frac{0.693}{\mu_{max}}$$

During the exponential phase of balanced growth, cells are undergoing primary metabolism, explicitly those metabolic pathways that are essential for growth of the cell. Industrial fermentations requiring maximum cell biomass production or the extraction of primary metabolites or their products, therefore, aim to extend this phase of growth, often via fed-batch or continuous culture techniques (continuous nutrient input with concomitant withdrawal of biomass suspension).

Following the exponential phase, cells enter a period of zero population growth rate, the stationary phase, in which the accumulated biomass remains relatively constant and the specific growth rate returns to zero. After prolonged periods in stationary phase, cells may die and autolyse (see below). The stationary phase has been defined as the ability of fungi to survive for prolonged periods (i.e. months) without added nutrients. In addition to nutrient deprivation, other physiological causes may promote entry of fungal cells into stationary phase including toxic metabolites (notably ethanol), low pH, high CO_2, variable O_2 and high temperature. During the stationary phase of unbalanced growth, cells are undergoing secondary metabolism, specifically metabolic pathways not essential for growth of cells but involved in the survival of the organism. The industrial production of secondary metabolic compounds such as penicillin and the ergot alkaloids therefore involves the controlled maintenance of cell populations within a stationary phase of growth.

Filamentous fungi tend to grow as floating surface pellicles when cultivated in static liquid culture. In agitated liquid culture, fungi either form dispersed filamentous growth, or form pellets of aggregated mycelium, subject to species, inoculum size, agitation rate and resource. Different growth forms will locally experience different microenvironmental conditions, which will affect the physiology and hence fermentation processes. In fungal biotechnology, cell morphology may directly influence fermentation progress. For example, the rheological properties of the growth medium, oxygen transfer and nutrient uptake may adversely affect bioproduct formation. In addition, in dimorphic yeasts such as *Kluyveromyces marxianus* filamentous cells with a large surface area may be better suited to immobilization compared with ellipsoidal unicellular yeast forms with a low surface area.

Yeast or fungal cell immobilization onto inert carriers has many advantages over free cell suspension culture in industrial processes. Cells may be successfully immobilized either by entrapment, aggregation, containment, attachment or deposition.

Fungal biofilms represent a natural form of cell immobilization resulting from cellular attachment to solid support materials. Yeast biofilms have several practical applications in fermentation biotechnology and are also medically important with regard to colonization of human tissue. In this latter case of pathogenic yeast biofilms, *Candida albicans* has been shown to adhere to surgical devices such as heart pacemakers and catheters, human epithelial cells and dental acrylic.

1.6.4 Fungal cell death

An understanding of the death of fungal cells is important from a fundamental viewpoint because fungi, especially yeasts, represent valuable model systems for the study of cellular ageing and apoptosis (programmed cell death). From a practical perspective, cell death in fungi is pertinent in relation to the following situations: industrial fermentation biotechnology (where high culture viabilities are desired), food preservation (regarding inhibition of spoilage fungal growth), food production (promotion of cellular autolysis for yeast extracts) and clinical mycology (where fungal death is the goal in treatment of human mycoses).

Numerous physical, chemical and biological factors influence fungal cell death, which may be defined as an inability of cells to reproduce. In relation to the former, fungi will die if confronted with excessive heat, extreme cold, high-voltage electricity, ionizing radiation or high hydrostatic or osmotic pressures. When the cells' physiological protection responses are insufficient to counteract the cellular damage caused by such physical stress, cells will die. In industrial situations, physical treatments can be used to eradicate contaminant fungi. For example, exposure of yeasts to elevated temperatures may lead to their thermal death, and this is exploited in the pasteurization of foods and beverages to kill spoilage yeasts.

There are numerous chemical factors influencing survival of fungi. Several external chemical agents are fungicidal, including toxic organic compounds, oxygen free radicals and heavy metals. Chemical preservatives are commonly employed as antifungal agents in foodstuffs, including weak acids such as sorbic, benzoic and acetic acids. These agents, which are generally fungistatic rather than fungicidal, act by dissipating plasma membrane proton gradients and depressing cell pH when they dissociate into ions in the yeast cytoplasm. Similarly, sulphur dioxide, which has long been used to eliminate undesirable yeasts (and bacteria) from wine, dissociates within the yeast cell to SO_3^{2-} and HSO_3^-, resulting in a decline in intracellular pH, and this forms the basis of its antizymotic action. Fungicidal acids include medium-chain fatty acids (e.g. decanoic

acid), which may cause the rapid fungal cell death of yeast by disruption of cell membrane integrity. Endogenous chemical factors such as ethanol and other toxic metabolites (e.g. acetaldehyde) produced by fermentative activity, excessive intracellular acidity or alkalinity, inability to protect against oxidative damage or sequester toxic metals, may also prove lethal to fungi. If fungal cells are unable to detoxify or counteract detrimental effects of chemicals, they may die.

Examples of lethal biotic interactions with fungi include direct ingestion (by insects, protozoa), engulfment and lysis (by mycoparasitizing fungi), direct predation (by haustoria-mediated processes) and intoxication (by killer toxin-producing yeasts). Killer yeasts are yeasts that secrete proteinaceous toxins that are lethal to other yeasts but to which the killers themselves are immune. Several yeast species have now been identified as possessing killer character, but the best known is the K1 system in *S. cerevisiae*. The K1 toxin from this species acts by binding to cell wall receptors in sensitive yeast cells, followed by plasma membrane channel formation. This latter event causes disruption of membrane permeability, which leads to the death of sensitive cells. Killer cells synthesize an immunity protein that prevents cellular suicide. In recent years, it has been established that some killer yeasts may also possess antimycotic effects against filamentous fungi. This has lead to the potential use of killer yeasts and their toxins as novel antifungal biocontrol agents for combating important fungal pathogens in agriculture.

With regard to endogenous biotic factors influencing fungal cell survival, several physiological, morphological, genetic and biochemical events may take place leading to 'self-inflicted' death. For example, fungal autolysis may be described as self-digestion of fungal cells and occurs when endogenous (vacuolar) hydrolytic enzymes, notably proteases and carbohydrases, cause dissolution of cytoplasmic proteins and cell wall polysaccharides, respectively. Autolytic enzymatic activity is encouraged during the production of yeast extracts in the food industry by using high temperatures (e.g. 45 °C), salt (to encourage plasmolysis) and solvents (to promote lipid dissolution). Exogenous hydrolytic enzymes such as papain can also be used to accelerate cell wall breakdown.

Genetic factors also influence fungal cell death. For example, cells may commit suicide following DNA damage, presumably to avoid the risk of producing genetically altered progeny. Cellular ageing and apoptotic cell death has been widely studied in yeasts, especially *S. cerevisiae*, which is a valuable model organism for understanding the molecular genetic basis of the ageing process in mammalian cells. Beyond a certain finite limit of cell division cycles (generally around 20 in *S. cerevisiae*), this yeast can generate no further progeny and cells enter a senescent physiological state leading to death. Aged and senescent populations of this yeast can be isolated, together with mutants displaying age-related phenotypes. In *S. cerevisiae*, *UTH* (yo*uth*) genes have now been identified, which appear to influence both stress resistance and longevity.

1.7 Conclusions

This chapter has highlighted the physiological biodiversity of yeasts and fungi in terms of morphology, growth, metabolism and cell death. Understanding the ways in which fungi interact with their growth environment is crucial for the control of fungal pathogens and in the industrial exploitation of yeasts and fungi in biotechnology.

1.8 Further reading

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1.9 Revision questions

Q 1.1 Describe the fine structure of the fungal cell envelope and explain its main physiological roles.

Q 1.2 Outline the main nutrients required for the growth of yeasts and fungi and indicate how such nutrients may be accumulated by fungal cells from their growth environment.

Q 1.3 Explain the fate of glucose metabolized (a) by fungi under aerobic growth conditions and (b) by yeasts under anaerobic growth conditions.

Q 1.4 Describe the major physical factors influencing the growth of fungal cells.

Q 1.5 Compare and contrast the modes of cellular reproduction in yeasts and fungi.

Q 1.6 For yeast cells, explain what is meant by exponential growth and describe how the doubling time of a yeast culture population may be determined.

2
Fungal Genetics

Malcolm Whiteway and Catherine Bachewich

2.1 Introduction

2.1.1 Fungi as pioneer organisms for genetic analysis

Genetic manipulation of organisms implies the ability to direct the formation of new combinations of traits within an individual. This process has historically been an important human endeavour, providing us with our breeds of domestic animals, our economically important plants and our industrially important fungi. Genetic manipulation can be as simple as identifying and selecting, within a population, rare individuals that contain interesting traits, but is made more powerful by the ability to enhance the rate of individual variation, and more powerful still when traits identified in different individuals can be combined. Consequently, mutagenesis and genetic recombination underpin the process of genetic manipulation. Such genetic manipulation can be used directly for practical ends (new varieties of tomatoes for example), or for more academic aims directed at an understanding of life.

Fungi were among the first organisms to be studied scientifically through genetics. Although peas and fruit flies provided the initial evidence for genes and for genetic linkage, some of the earliest fundamental insights into the genetic structure of organisms came from pioneering studies in fungal systems. One exceptional insight developed from analysis of fungal systems, in this case *Neurospora crassa*, was the recognition that individual enzymatic functions were encoded by the information from individual genes, a result rewarded by the 1958 Nobel Prize in Physiology or Medicine to G. Beadle and E. Tatum. Other fundamental advances based on the genetic analysis of fungi included the

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dissection of the cell cycle of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, work that led to the 2001 Nobel Prize in Medicine to L. Hartwell and P. Nurse. This award, over 40 years after that to Beadle and Tatum, shows that fungal systems have maintained their utility in uncovering important biological truths.

More recently, large-scale, semi-industrialized international efforts had an enormous impact on the field of fungal genetics. These efforts have been at the forefront of the development of the science of genomics. Many of the primary successes of genomics have come through studies on fungi, in particular the baker's yeast *Saccharomyces cerevisiae*. Efforts directed at the analysis of the yeast genome have provided the first sequence of a eukaryotic chromosome, the first sequence of an entire eukaryotic genome and the first development of a systematically created collection of null mutants of all the genes of an organism. Building on this pioneering work, the sequencing of many fungal genomes is now underway, and many fungal genome sequences are now fully completed or available as unannotated draft sequences.

2.1.2 Significance/advantages of fungi as model organisms

Fungi are among the pre-eminent models for the genetic investigation of basic cellular processes. There are many intrinsic characteristics of fungal systems that make them ideal model organisms for genetic studies. As unicellular organisms that can grow in simple defined media, they are easy to culture. Because they often contain a stable, propagatable haploid phase they are easy to mutate, and in those organisms with a well characterized sexual cycle the mutants can be readily combined. Finally, because they are eukaryotic cells, they exhibit many of the properties and functions characteristic of human cells, and thus served as a better model for many cellular processes than the bacterial systems that had been investigated in depth previously.

The advent of the molecular biological revolution in the 1970s strengthened the importance of fungal systems as models for genetic studies of eukaryotic cell function. Because each individual fungal cell was autonomous, transformation with external DNA allowed the efficient genetic engineering of an entire organism. The rapid development of fungal transformation systems after the initial successes with *S. cerevisiae*, followed by the construction of efficient vectors for the transfer of genes and the cloning of the various components of chromosomes, allowed for effective genetic manipulation of fungal cells. Ultimately, this technology permits the construction of strains of fungi genetically designed to differ from a standard strain by as little as a single selected nucleotide.

This chapter provides an overview of various aspects of fungi and their use as model organisms for genetic analysis. Representative species from the higher fungi, including ascomycetes and basidiomycetes, comprise the focus for dis-

cussion, and comparisons between the yeasts (*Saccharomyces cerevisiae*) and the filamentous organisms (*Neurospora crassa*, *Aspergillus nidulans*, *Coprinus cinereus*) are emphasized to introduce both the advantages of particular systems and the diversity within the kingdom. Examples of dimorphic fungi, which live in both a yeast and filamentous form, are also discussed to highlight unique features and variations on themes within the fungi.

2.2 Fungal life cycles

2.2.1 Ascomycete yeast (*Saccharomyces cerevisiae*)

S. cerevisiae is an extremely well studied organism, with a clearly defined and experimentally manipulable life cycle. The life cycle of yeast involves mitotically propagating haploid forms of two distinct mating types, and a diploid form that can either grow vegetatively or be induced into a meiotic developmental pathway through manipulation of the nutrient conditions of the growth medium. The cellular pathways regulating processes such as mitotic proliferation, cell recognition and mating, meiosis and sporulation have been extensively studied on a molecular level, and are generally well understood.

Mitotic growth of yeast cells involves budding (Figures 2.1, 2.6). During this process growth of the cell is directed to a specific location on the surface of the mother cell, and a new cell is formed somewhat like blowing up a balloon through a hole in the mother cell. This involves highly polarized growth of the developing daughter cell, implicating both the actin and microtubule-based cytoskeletal networks, and is tightly coordinated with the cell cycle. This coordination ensures that the daughter cell receives a complete copy of the genetic material. Both haploid and diploid cells divide by the budding process, although there are subtle differences in the choice of the sites of bud emergence between haploids and diploids. In addition, some diploid cells can also modify the coordination of the cell cycle and polarized growth to switch to a pseudohyphal growth mode. In this growth pattern individual cells are more elongated, and the budding pattern leads to the formation of chains of cells rather than compact colonies characteristic of the true budding mode.

Genetic analysis is highly developed in *S. cerevisiae*. When vegetatively growing haploid cells of opposite mating types are brought into proximity, they communicate to each other by diffusible pheromones, synchronize their cell cycles, conjugate and then fuse their nuclei to create non-mating, meiosis-proficient diploids. These diploids can be identified visually in their initial zygote form, and separated from the haploids by micromanipulation, or identified selectively because they contain a pattern of genetic traits not possessed by either haploid parent.

Under rich growth conditions such diploid cells propagate vegetatively, but under conditions of nitrogen and fermentable carbon limitation the diploid

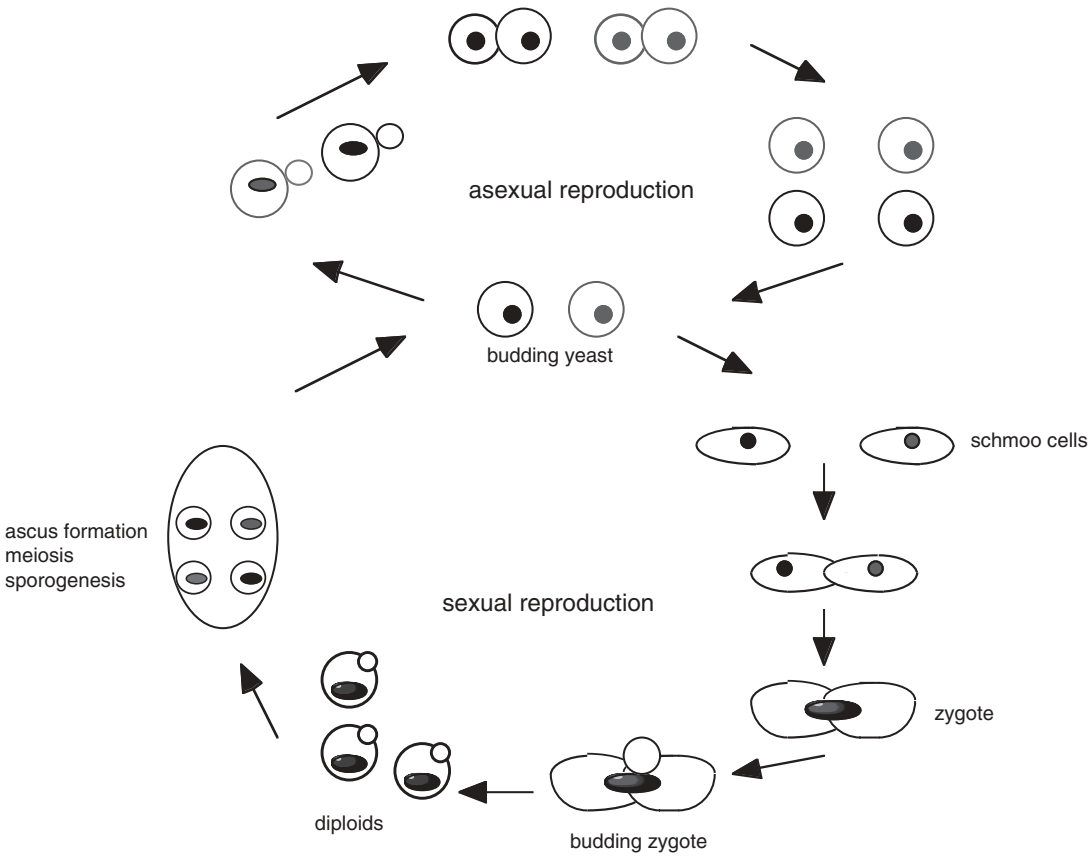

Figure 2.1 Life cycles of *Saccharomyces cerevisiae*

cells are induced to initiate meiosis and sporulation. The ability to propagate the diploid allows the amplification of the initial mating product, and provides an essentially unlimited source of potential meiotic events from a single mating.

2.2.2 Ascomycete filamentous fungi (*Neurospora crassa* and *Aspergillus nidulans*)

The filamentous fungi differ from the yeasts in that they grow vegetatively as hyphae, which are highly polarized filaments that extend indefinitely at their tips. The hypha initiates new tips in the form of branches from sub-apical regions, and together the growing mass constitutes the mycelium. Hyphae are predominantly multinucleated, with cross-walls called septa dividing the

hypha into compartments. The compartments are connected through pores in the septa, and therefore display cytoplasmic continuity. The function of the hypha is primarily in nutrient acquisition, exploration of the environment and secretion of enzymes to assist in both processes. In pathogenic fungi, the hyphal growth form can also be important for virulence. In filamentous fungi, vegetative hyphal growth initiates from a spore. Spores are products of either sexual (ascospores, basidiospores) or asexual (conidia) reproduction. Conidia are typically produced from a differentiated structure called a conidiophore, whereas ascospores and basidiospores are produced within an ascus or basidium, respectively, contained with the fruiting body called an ascocarp or basidiocarp.

During asexual reproduction in the ascomycetes, such as *Aspergillus nidulans* (Figures 2.2, 2.6), a spore containing a single nucleus (monokaryotic) germinates into a mutinucleate, homokaryotic hypha. The hypha grows and develops branches for a period of time, then initiates a specialized branch called the conidiophore. Development of the conidiophore involves numerous different cell types, and is investigated as a model developmental process. The nucleus divides mitotically within the conidiophore, allowing the ultimate production of asexual, haploid conidia. Upon release, conidia germinate into vegetatively growing hyphae, and the cycle continues. The factors that trigger initial conidiophore development in *Aspergillus* are not clear, but involve the supply of carbon and nitrogen. The process can normally only occur in cultures grown on solid media with an air interphase; conidiation does not occur in liquid.

Sexual reproduction in *Aspergillus nidulans* begins when vegetatively growing hyphae fuse to create a heterokaryon, or dikaryotic hypha (Figure 2.2). The dikaryotic hyphae differentiate into a developing fruiting body called a cleistothecia. The fruiting body is a complex structure composed of many cell types, including both sterile and fertile hyphae. The dikaryotic fertile hyphae within the cleistothecium develop into hooked structures called the croziers, which then differentiate into developing asci. Karyogamy or nuclear fusion occurs within the crozier, creating a diploid. The diploid quickly undergoes meiosis and the four meiotic products undergo mitosis, creating eight haploid ascospores. The ascospores undergo another round of mitosis and are thus binucleate. Thousands of asci are contained within a cleistothecium and are fragile, preventing their individual isolation. Upon release, the ascospores germinate into hyphae as described. *Aspergillus* is homothallic, or self-fertile, and sexual reproduction can be initiated within one colony containing genetically identical nuclei. In the absence of heterokaryon formation with another strain, the individual strain differentiates a cleistothecium as described, into which the hypha develops into a crozier and ascogenous hypha. Unlike *Saccharomyces cerevisiae*, *Aspergillus nidulans* does not undergo any mating-type switching. *Aspergillus nidulans* hyphae can also grow as heterokaryons and diploids as part of a parasexual cycle, which will be discussed later in the chapter.

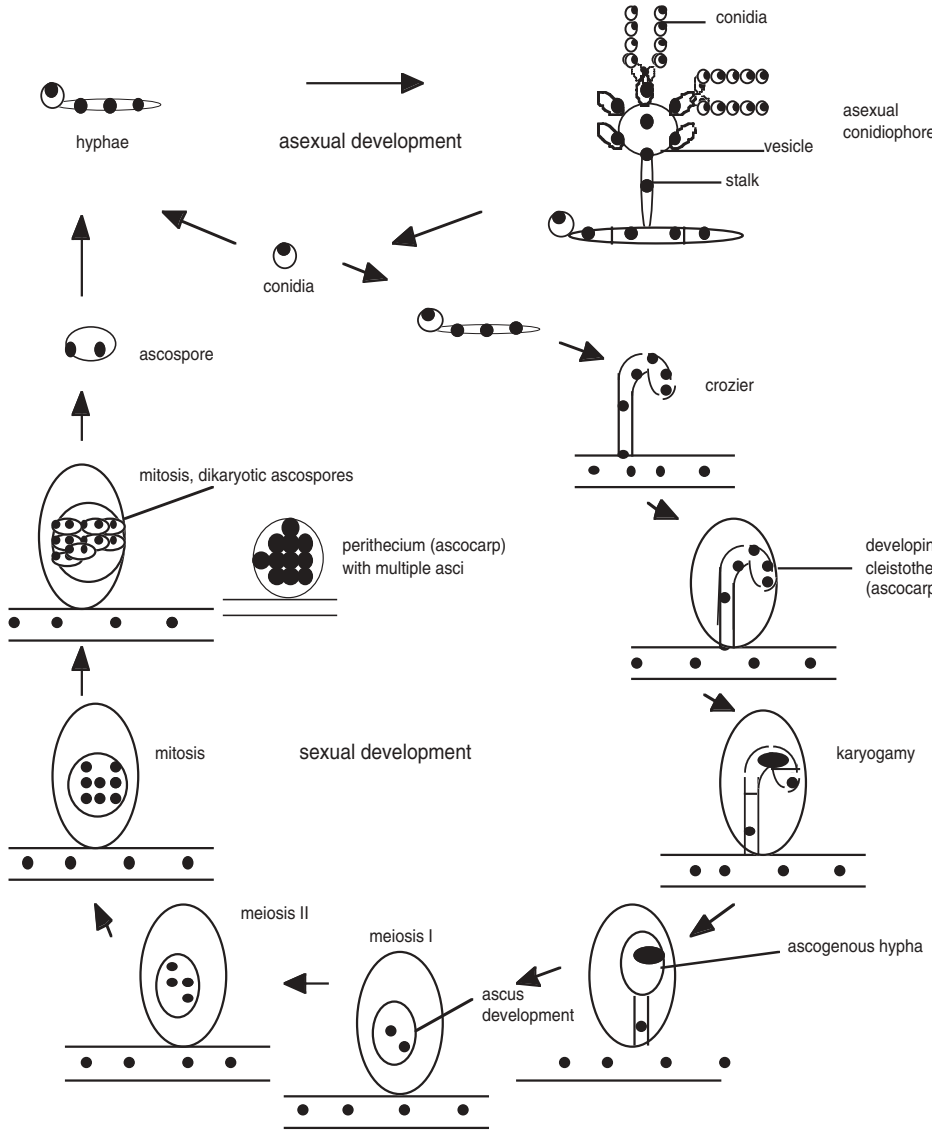

Figure 2.2 Life cycles of *Aspergillus nidulans*

In *Neurospora crassa* (Figure 2.3), asexual reproduction is triggered by circadian rhythyms, or an internal clock mechanism, and produces both macro- and microconidia. Macroconidia are produced first from aerial hyphae, and are used for subculturing strains, while microconidia are produced later in the growth process and are not very viable. Macroconidia germinate into vegetatively growing hyphae, but also serve a function during sexual reproduction.

The sexual cycle is initiated in response to nitrogen starvation, or changes in temperature or light. *Neurospora crassa* is heterothallic, and therefore requires

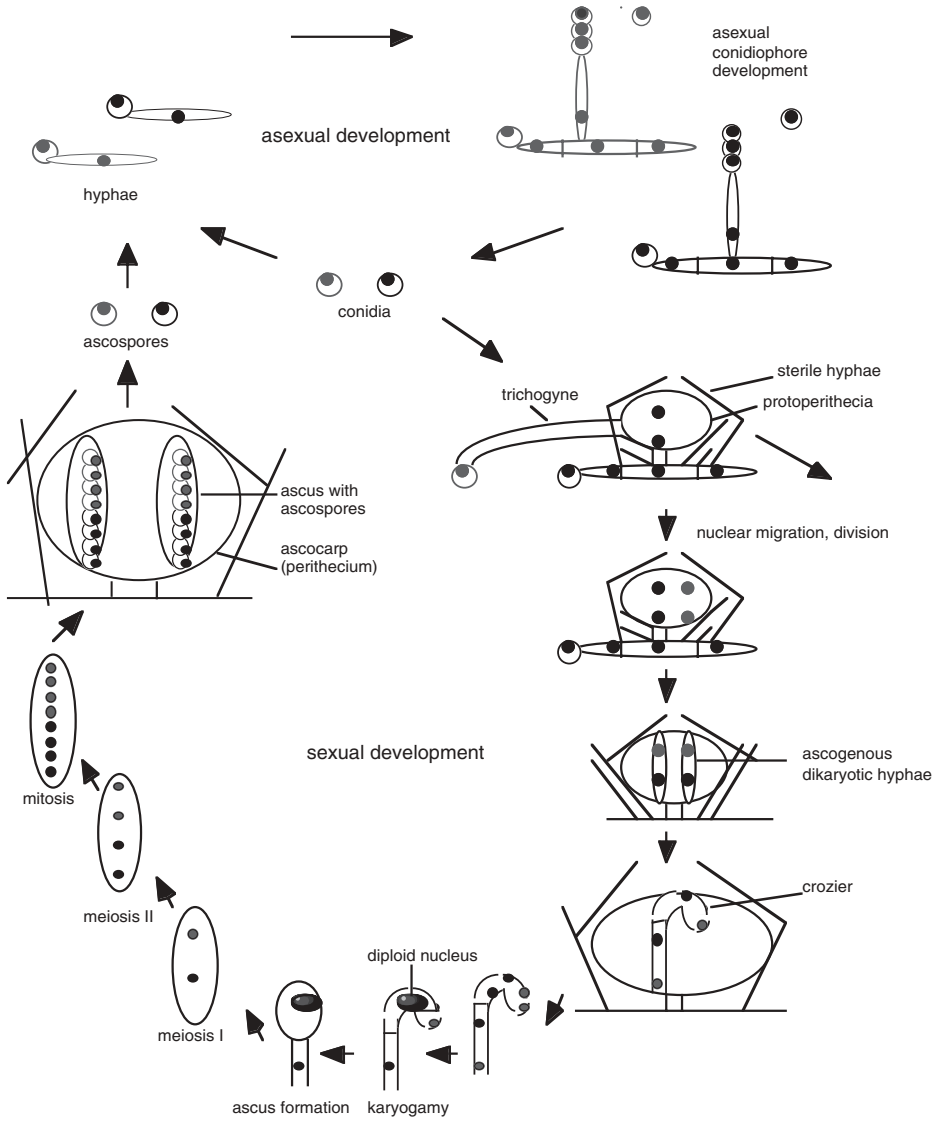

Figure 2.3 Life cycle of *Neurospora crassa*

genetically different mating partners. Macroconidia or microconidia produced from hyphae serve as the 'male' and produce a pheromone, which is a hydrophobic peptide. The opposite strain serving as the female develops a fruiting body intermediate called a protoperithecia. A polarized structure called a trichogyne grows from the protoperithecium of one mating-type female towards and fuses with the male conidia of the opposite mating type. The nucleus from the latter moves through the trichogyne into the ascogonium within the protoperithecium, which is then referred to as the perithecium. Nuclei from both mating partners

divide, within a developing dikaryotic ascogenous hyphal structure. The ascogenous hypha develops a crozier, where nuclear fusion or karyogamy takes place, followed quickly by meiosis within the developing ascus. Mitosis and subsequent ascosporogenesis results in eight spores within an ascus within the perithecium. Acsi are long and slender in *Neurospora,* allowing for individual dissection and separation of ordered ascospores.

2.2.3 Basidiomycete filamentous fungi (*Coprinus cinereus*)

The basidiomycete life cycle is typically similar to the ascomycetes with a few exceptions. In *Coprinus cinereus* (Figure 2.4), a typical mushroom fungus, monokaryotic hyphae produce asexual spores called oidia, which germinate and form hyphae. To initiate sexual reproduction, monokaryotic hyphae fuse at their tips (anastomosis) to create a dikaryotic hypha. The dikaryotic hypha grows vegetatively, and is distinguished from hyphae of ascomycetes by the presence of hooked cells or clamp connections, which connect tandem septated compartments of the hypha. Changes in temperature and light can trigger the hypha to undergo differentiation into the fruiting structure, or basidiocarp. Within the basidiocarp, basidium formation (the equivalent of an ascus) and karyogamy take place. Meiosis produces the basidiospores, which hang off the basidium contained within the gills of the mushroom cap, as opposed to being encased as in the asci in ascomycetes. Haploid basidiospores are then released, and germinate into monokaryotic hyphae and continue the cycle.

In dimorphic basidiomycetes that exist in both yeast and hyphal forms, such as the human pathogen *Cryptococcus neoformins* (Figure 2.5), vegetative growth occurs via budding yeast. Sexual reproduction involves the differentiation of yeast cells into hyphae upon exposure to pheromone, resulting in dikaryotic hyphae. Basidia differentiate from the ends of the hyphae, in which karyogamy followed by meiosis and mitosis occurs, producing haploid basidiospores. Upon release, the spores grow as budding yeast. The yeast cells can also undergo asexual sporulation in response to nitrogen limitation or drying. Under these conditions, yeast cells differentiate into hyphae, which differentiate monokaryotic basidia at their tips. Mitosis occurs within the basidia, producing haploid spores, which then germinate into yeast cells.

Filamentous fungi exhibit many variations in their life cycles, and some do not exhibit a known sexual phase (the deuteromycetes). Others exploit parts of the life cycle for pathogenesis, particularly in the pathogenic basidiomycetes. For example, the dimorphic corn smut fungus *Ustilago maydis* exists as a nonpathogenic yeast form, but upon mating yeast cells differentiate into dikaryotic hyphae, which are associated with virulence.

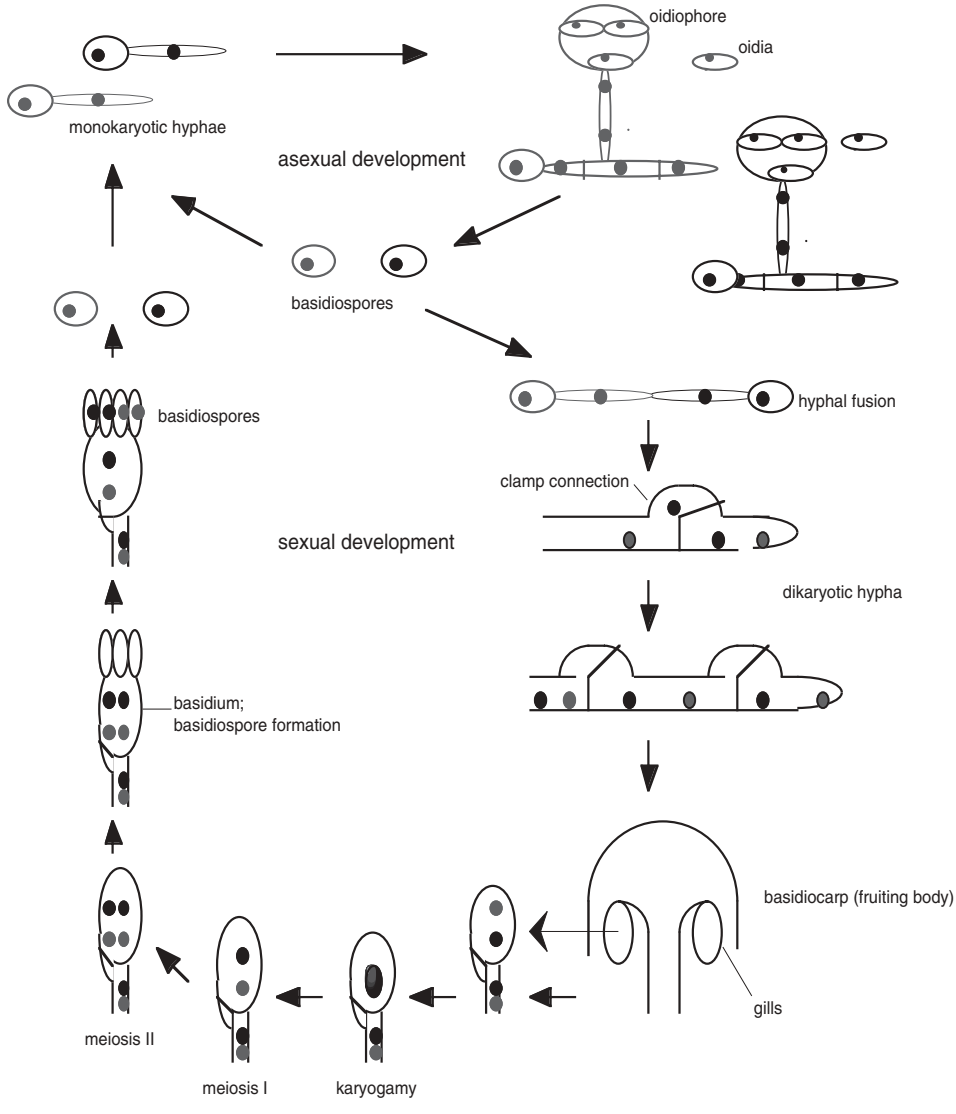

Figure 2.4 Life cycles of *Coprinus cinereus*

2.3 Sexual analysis: regulation of mating

2.3.1 Ascomycete yeast

The yeast *S. cerevisiae* has a simple mating system, with cells of two haploid mating types termed **a** and α. These cells can conjugate to form a diploid cell containing both **a** and α information. The **a**/α diploid is not capable of mating but can initiate meiosis to form four haploid products, two of which are mating

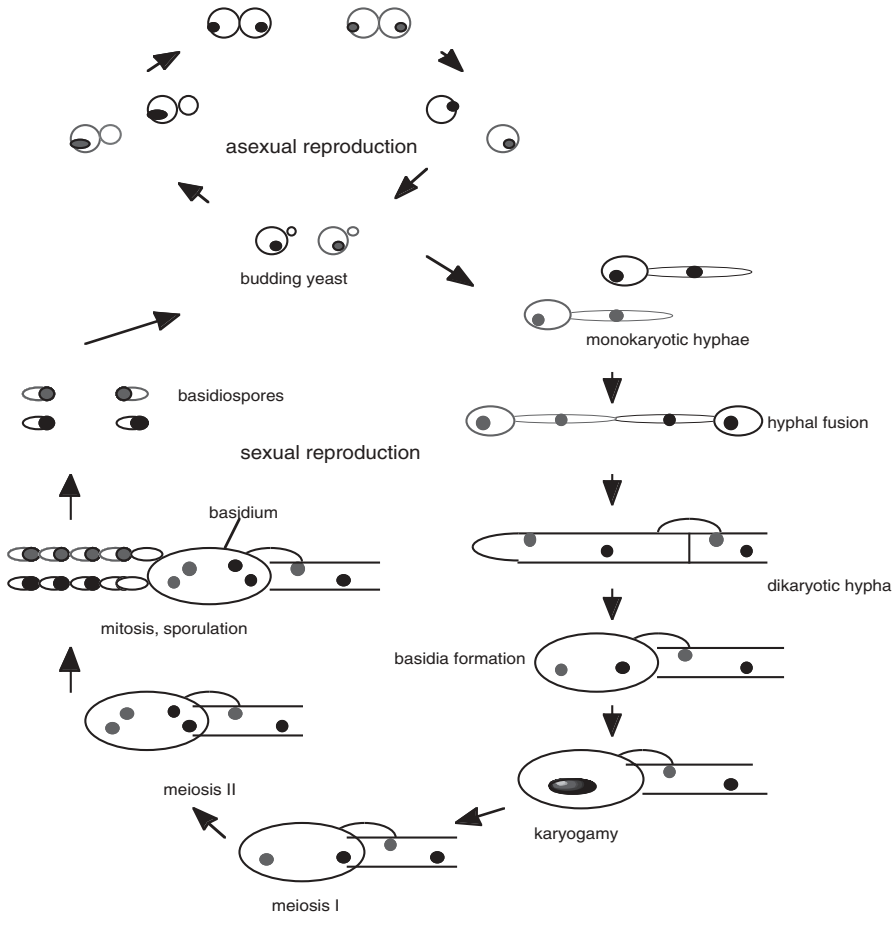

Figure 2.5 Life cycles of *Cryptococcus neoformins*

type **a** and two of which are mating type α. Laboratory strains typically have stable mating types, and are termed heterothallic. In contrast, most wild strains are homothallic, and do not have stable mating types. Instead, during mitotic growth the cells are capable of switching their mating types, and thus a growing spore colony develops cells of both **a** and α. These cells mate with each other, resulting in a colony that grows up as an **a**/α diploid. The regulation of the stability of the mating type is controlled by a single locus, termed the homothallism locus (*HO*). The *HO* locus contains a functional endonuclease, while the stable mating types of the heterothallic strains result from a defective allele of this locus designated *ho*.

The reason that an endonuclease controls the stability of the mating type is the result of a sophisticated genetic exchange system involving expressed and

unexpressed cassettes of information. The mating type of the cell is controlled by a single locus termed *MAT* close to the centromere of chromosome 3. When this locus contains the *MAT***a** allele the cells are of the **a** mating type; when the locus contains the *MAT*α allele the cells are mating type α. If there is no information at *MAT* the cells select **a** as the default mating type. However, the typical *S. cerevisiae* cell also contains an extra copy of both mating type genes, typically **a** information near the right telomere of chromosome 3 at a locus called *HMR*, and α information at the left telomere of chromosome 3 at locus *HML*. The sequences at *HML* and *HMR*, although structurally identical to the sequences for **a** and α information at *MAT*, are not expressed due to the action of a series of proteins designated silent information regulators. In the presence of a functional *HO* endonuclease the information is exchanged between the *HMR* or *HML* loci and the *MAT* locus as often as once per cell division. This exchange is recombinational; the *HO* endonucleases makes a double-strand cut at the *MAT* locus, and gene conversion then transfers the information from one of the silent loci to the *MAT* locus. The same machinery that keeps the information at *HML* and *HMR* unexpressed blocks the cutting of these DNA sequences by *HO*, so the informational exchange is typically unidirectional.

The *MAT* locus defines the mating type of the cell through direct transcriptional control. Each allele, *MAT***a** and *MAT*α, expresses transcription factors, and these transcription factors control the expression of blocks of genes defining the two cell types. *MAT*α encodes two transcription factors, α1 and α2. The role of α1 is to stimulate the expression of α-specific genes such as *STE3*, encoding the receptor for the **a**-factor mating pheromone, and the genes specifying the α-factor mating pheromone. The α2 transcription factor is a repressor, serving to repress **a**-specific gene expression in the *MAT*α cells, and together with the **a**1 factor to shut off haploid gene expression in *MAT***a**/*MAT*α diploid cells. Among the key genes shut off in the diploid cell is *RME1*, which encodes a repressor of meiosis; this regulatory circuit ensures that it is the **a**/α diploid cell that is uniquely capable of meiosis and sporulation.

2.3.2 Filamentous ascomycetes

Neurospora crassa is heterothallic and requires two mating types, A and a, for sexual reproduction. The mating-type loci, *mat A* and *mat a,* control gene expression required for the mating process. The DNA sequences at the mating loci of opposite mating types are very different, and are therefore regarded as 'idiomorphs' as opposed to alleles. The *mat a* idiomorph is 3.2 kb in length, and encodes one gene called *mat a-1*, while the *mat A* idiomorph is 5.3 kb long and encodes three genes, including *mat A-1*, *A-2* and *A-3*. The *mat a-1* and *mat A-3* genes encode for HMG box-containing DNA binding proteins, and are the major regulators of mating in both strains. Homologues of such factors are

required for mating in other organisms, including some filamentous fungi and the fission yeast *Schizosaccharomyces pombe*. The *mat A-1* product is also a DNA-binding protein and similar to α1p from *Saccharomyces cerevisiae*. *mat A-3* encodes a protein with little homology in other organisms and of unknown function, but togther with *mat A-2* is required for ascosporogenesis. *mat A-2* and *A-3* are expressed constituitively during both vegetative growth and sexual development. In contrast to that in *Saccharomyces cerevisiae*, the downstream targets of the mating regulatory genes are unknown. They presumably control expression of the mating pheromone, but other processes must also be regulated, including nuclear migration, nuclear compatibility and fruiting body development. Since filamentous fungi utilize multiple different cell types for sexual reproduction and contain complex fruiting body structures, including different thalli for male and female mating partners, the regulation and function of the mating type loci must be more complex than in yeast. In contrast to *Saccharomyces cerevisiae*, mating type switching does not occur in *Neurospora crassa*.

The mating loci in *Neurospora* regulate additional processes, including vegetative, heterokaryon compatibility. Vegetative hyphae can fuse to form a heterokaryon, but only if they arise from opposite mating type strains. If hyphal fusion occurs between incompatible cells, the fused hyphal compartment seals off from the rest of the hyphae through deposition of cross-walls, and the compartment undergoes a type of programmed cell death characterized by DNA fragmentation, organelle and cytoplasmic breakdown and vacuole production. Mutants of the *mat a* strain which lost the incompatibility response were affected in the *mat a-1* open reading frame (orf), and analysis of the *mat a-1* protein identified domains important for mating versus vegetative incompatibility.

Several filamentous fungi, including *Aspergillus nidulans*, are homothallic or self-fertile, where a colony derived from a single spore is able to undergo sexual reproduction. Although the genetic basis of homothallism is not understood, recent completion and analysis of the genome from *Aspergillus nidulans* has uncovered the presence of many conserved elements of mating from heterothallic species, suggesting that sexual reproduction may be regulated by similar genes in 'selfing' fungi. For example, MAT-2 and MAT-1 genes encoding an HMG box-containing DNA-binding protein and an α1p domain homologue, respectively, have now been identified. In addition, genes encoding homologues to the hydrophillic pheromone alpha factor in *Saccharomyces*, the mating pheromone protease *KEX2* and pheromone receptors *STE2* and *STE3* are present in the genome. However, no homologue to **a**-factor mating pheromone was detected, despite the fact that its receptor was present. Whether these elements are required for mating in this self-fertile organism, or simply reflect evolution of homothallism from a heterothallic progenitor species, remains to be determined.

2.3.3 Filamentous ascomycete dimorphic fungi

Candida albicans (Figure 2.6) is an important human pathogen and has been extensively studied as a result. *C. albicans* had been classified as an asexual deuteromycete, but recent genomic studies have provided convincing evidence for the potential for a sexual cycle. However, although a well defined mating system has been identified that allows the conjugation of mating-type locus homozygous diploid cells, there is currently no evidence for a functional meiotic pathway that allows reductional division and a return to the diploid state from the tetraploid.

The detection of the potential mating ability of *C. albicans* arose through analysis of the genome sequence. A region of the genome was detected that encoded genes similar to those found at the mating-type locus of *S. cerevisiae*. Further analysis of this region uncovered a more complex locus than that found in *S. cerevisiae*; in addition to the candidate cell-type regulating transcription factors, there were other genes whose function implied no obvious link to the mating process. Such complex loci are found in other fungi, such as *Cryptococcus neoformans*, and in algae such as *Chlamydomonas reinhardtii*; analysis

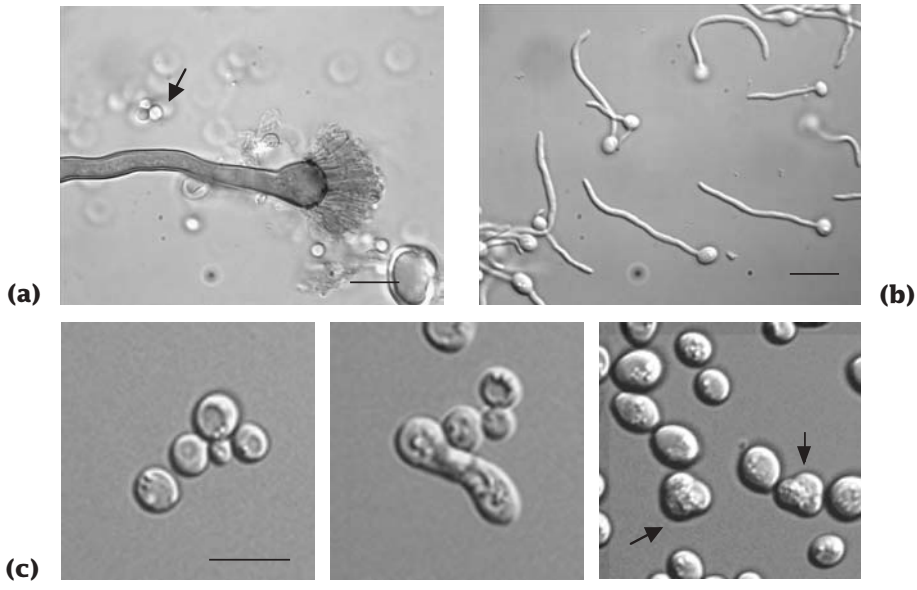

Figure 2.6 (a) Developing conidiophore composed of a vesicle giving rise to phialides and conidia in the filamentous fungus *A. nidulans*. Released conidia are indicated by the arrow. (b) Hyphae growing from yeast cells of the dimorphic fungus *C. albicans*. (c) Various stages in the life cycle of the yeast *S. cerevisaie*. The first panel demonstrates vegetatively growing yeast. The second panel shows a zygote, and the third panel demonstrates asci (arrows). Bars: 10 μm

across the spectrum of mating-proficient lower eukaryotes suggests that many organisms contain mating-type loci that mix genes with roles in controlling the mating type with genes involved in other cellular processes. In *C. albicans* these genes include oxysterol-binding proteins (*OBPa* and *OBP*α), poly-A polymerases (*PAPa* and *PAP*α) and phosphatidyl inositol kinases (*PIKa* and *PIK*α). The a and α versions of each protein are somewhat divergent, but the ability to homozygose each *MTL* allele shows that both versions of the proteins are capable of supporting cell viability.

In contrast to *S. cerevisiae*, where the *MATa* locus expresses one transcription factor and the *MAT*α locus encodes two factors, both the *MTLa* and *MTL*α loci of *C. albicans* express two transcription factors. Genetic analysis suggests that *MTLa2*, an HMG box containing protein, is a positive effector of *MTLa* functions. This positive function (and gene) is missing from *S. cerevisiae* cells, where the *MATa* phenotype is expressed as the default state in the absence of any *MAT* information. This makes the *C. albicans MTL* loci structurally more complex than the *S. cerevisiae* loci, but the mating-type regulatory circuit is actually more direct. In *C. albicans* the *MTLa* locus expresses *MTLa2*, which directs a mating functions, and the *MTL*α locus expresses *MTL*α*1*, controlling α mating functions. In the diploid state the other transcription factors, Mtla1p and Mtlα2p, combine to repress mating functions, as well as white–opaque switching. Thus each locus has both positive and negative roles within the mating-type circuit.

The link between mating ability and the phenomenon of white–opaque switching represents another detail of the *C. albicans* mating process. White–-opaque switching was identified as a high-frequency event occurring in some *Candida* cells through which cells changed their morphology, some of their physiological characteristics and their colony morphology. It is now appreciated that the ability to undergo this switching was occurring in cells that had homozygosed their mating type, thus relieving the repression of the switching process caused by the a1/α2 regulatory molecule. Efficient mating in *Candida* cells requires that the cells be in the opaque state, rather than the white state, but the molecular mechanism underpinning this relationship is not yet understood.

2.3.4 Filamentous basidiomycetes

The basidiomycetes are novel in that they have multi-allelic mating-type genes. In *Coprinus cinereus*, for example, more than 12 000 mating types exist, demonstrating the diversity in mating systems and complex levels of control within the filamentous fungi. *Coprinus* has two unlinked mating-type loci, A and B, which are polymorphic and contain sub-loci called α and β. The mating system is therefore described as being tetrapolar. The α and β loci are redundant, but recombination occurs between them. Together these loci contribute hundreds of specificities to A and B loci, creating thousands of different mating types.

The A locus encodes genes for homeodomain proteins, and regulates nuclear pairing, clamp connection formation and septation. At Aα, two classes of homeodomain proteins are produced. The HD1 and HD2 classes contains homologues to α2p and **a**1, respectively, from *Saccharomyces cerevisiae*. HD1 and HD2 proteins from different specificity loci dimerize and subsequently regulate the dikaryon. The B locus encodes six pheromone genes and three pheromone receptor genes. In contrast to ascomycetes, where pheromone signaling stimulates the formation of mating-specific morphological shapes, pheromone signaling in *Coprinus* stimulates fusion of the monokaryotic hyphae to initiate the sexual cycle. The B locus also regulates nuclear migration and attachment between the clamp connection and the corresponding supapical hyphal compartment. As in the filamentous ascomycete *Neurospora crassa*, the targets of the mating loci genes are currently not well characterized.

2.4 Unique characteristics of filamentous fungi that are advantageous for genetic analysis

2.4.1 Parasexual analysis

Parasexual genetics involves examination of recombination in the absence of sexual reproduction, and has been helpful in mapping genes to chromosomes. The unique feature of heterokaryosis, or maintaining two genetically distinct nuclei within one thallus in filamentous fungi, allows for this type of analysis. The parasexual cycle has been extensively utilized in *Aspergillus nidulans*, and involves heterokaryon formation, followed by karyogamy to produce a diploid that then undergoes spontaneous mitotic recombination. Thus genetic recombination can occur within the vegetatively growing hypha. Diploids can be differentiated from heterokaryons in parasexual analysis in *Aspergillus* based on spore colour. Heterokaryons formed from fusion of strains containing white or yellow spores will produce conidia of either colour. Sections of the colony that undergo karyogamy and form a heterozygous diploid, however, can be recognized by the resulting spore colour green, since the recessive mutations in spore colour leading to white and yellow spores will complement each other. The diploid is then isolated and forced to undergo haploidization through treatment of drugs, such as benomyl, to induce chromosome loss, and the resulting haploid products are analysed for evidence of mitotic crossing over. In *Aspergillus nidulans*, master strains containing unique markers per chromosome are used as a reference during 'crossing' with the test strain.

Parasexual analysis has recently been utilized to great advantage in *Candida albicans*, a diploid pathogen that does not have a known sexual phase involving meiosis, and therefore could not be analysed through traditional genetic analysis involving crossing of strains and sexual reproduction. *Candida albicans*

can mate and contains the necessary mating genes, as described earlier, but the resulting tetraploid products of mating break down to diploids through spontaneous chromosome loss, not meiosis. Therefore, *Candida albicans* appears to use a parasexual cycle to produce recombinant individuals. Parasexual analysis has been used for genetic linkage and construction of new strains in this organism, and holds promise for future mutagenic analysis.

2.4.2 Gene silencing

A unique feature that has greatly facilitated genetic/molecular analysis in the filamentous fungus *Neurospora crassa* is the process of repeat-induced point mutation, or RIP. If more than one copy of a gene is introduced in tandem into the haploid prior to sexual reproduction, the tandem copies are inactivated through GC to AT mutations when passed through the sexual cycle. Therefore, gene inactivation can be achieved by simply introducing additional copies, and allowing the strain to undergo sexual reproduction. In another process unique to *Neurospora crassa*, called quelling, genes that are introduced at heterologous locations are silenced. This silencing involves degradation of mRNA through several factors including an RNA-dependent RNA polymerase, whose homologues in other organisms are involved in the similar silencing process called RNA interference (RNAi). RIPing and quelling are therefore very useful for investigating gene function in *Neurospora crassa*, and for understanding the related mechanisms of gene silencing in other eukaryotes, including plants and worms.

2.5 Genetics as a tool

2.5.1 Tetrad analysis

Saccharomyces cerevisiae

The classic strategy for genetic analysis of *S. cerevisiae* meiosis involves tetrad analysis. Each meiotic event from a typical diploid cell generates four haploid spores. These spores are arranged in an ascus sac that is degraded by enzymatic treatment with an endoglucanase. This liberates the spores, which are then placed in separate locations on rich medium plates by micromanipulation and allowed to divide to form a spore colony. The spore colonies are analysed to determine the distribution of the markers introduced into the initial cross. Recombination occurs when the diploid undergoes meiosis. During meiosis I, chromosomes from each parent pair up, then duplicate, creating two chromatids that remain attached. Chiasma formation between paired homologous chro-

mosomes at this stage results in recombination of genetic material. Homologous chromosomes then separate to opposite poles of the meiotic spindle, and nuclear division results in two diploid nuclei. Independent assortment of chromosomes occurs at this stage. Meiosis II follows, which involves the splitting of the attached chromatids to opposite poles, and another round of nuclear division, resulting in four haploid nuclei. A critical advantage of the yeast meiotic process for genetic analysis is that all four products of the meioses are detected and available for analysis. This avoids questions of statistics in the analysis of segregation patterns, and allowed for the identification of 'non-Mendelian' segregation patterns characterized by a 3:1 rather than 2:2 distribution of heterozygous markers. The recombinational replacement of information from one allele to the other that generates this pattern is termed gene conversion.

Because there is no pattern to the position of the spores within the ascal sac, *S. cerevisiae* does not provide ordered tetrads as are found in some of the filamentous fungi. However, the identification of markers tightly linked to centromeres permits the indirect ordering of the spores relative to the actual meiotic event, because the centromere, and therefore all markers tightly linked to the centromere, segregates in the first meiotic division.

Selected tetrads

A modification of the classic tetrad analysis involves selected tetrads. This approach has been used during the analysis of meiotic recombination within a single locus. The frequency of intra-allelic recombinants is low, so selection is used to identify those infrequent meiotic events where a recombination has taken place. Typically such intragenic recombination studies involve heteroalleles of an auxotrophic marker, so all the non-recombinant products are auxotrophs. In contrast to standard tetrad analysis, the glucanase-treated ascal sacs are not spread on plates containing a rich growth medium, but rather are spread on plates that lack the nutrient required by the auxotrophic cells. The separated ascal groups are monitored microscopically to detect sets in which at least one member of the tetrad begins to germinate. These tetrads are then micromanipulated to permit subsequent analysis of the genetic structure of all four spores – this approach enriches for tetrads in which a recombination event is known to occur within the gene under study. This allows for a fine-structure analysis of the recombination process, and the sophisticated ability to monitor all the consequences of the meiotic events has been critical to the development of models of the process of meiotic recombination.

Random spore analysis

In situations where the pattern of marker segregation in individual asci is not important, random spore analysis can be applied. In this approach populations of asci are digested with glucanase *en masse*, and the mixture of haploid meiotic

products and unsporulated diploid cells is spread on plates. These plates are designed to select against the initial diploid cells, typically by demanding a recessive drug resistance marker that is only uncovered in the haploid cells. These haploid segregants can be rapidly screened to identify a cell containing a desired combination of markers, or the population can be scored to determine the overall patterns of segregation. An interesting recent development of the random spore strategy is found in the synthetic genetic array (SGA) approach pioneered by C. Boone and collaborators. In this approach, whole-genome-wide screens for synthetic lethal interactions are created by robotic replica plating, and sophisticated use of mating-type-specific gene expression is used to permit the growth of only a desired haploid cell type.

Neurospora crassa

Neurospora crassa is the pioneering organism for genetic analysis in microorganisms, predating work with bacteria and with the yeast *Saccharomyces cerevisiae*. It is an attractive model genetic system, since it is haploid and asci are large enough to remove ascospores, allowing the recovery of all products of meiosis and determining recombination of the parental genes within the progeny. *Neurospora* is particularly attractive for tetrad analysis since the ascospores are ordered within the linear ascus, allowing genes to be mapped in relation to the centromere. In *Neurospora crassa*, meiosis is followed by another mitotic division, producing eight haploid nuclei, or four pairs of sister nuclei. Sporogenesis then produces eight ascospores within the ascus (Figure 2.7). As in *Saccharomyces*, the individual spores are isolated and tested for traits such as nutrient requirements. Alternatively, spores from many asci are collected and analysed as a random sample.

Due to independent assortment of chromosomes at meiosis I, if two strains that differ at loci A and B mate (A/b × a/B), and A and B are located very close together, the chances of recombination between these loci is small, and the resulting meiotic products will be 50 per cent parental type (A/b and a/B) (Figure 2.7). Such close genes are described as being linked. Genes that are unlinked are far apart, and thus can recombine. The proportion of recombined products increases the further apart the genes, to 50 per cent. For example, if A and B loci were far apart or on different chromosomes, recombination could result in 25 per cent each of the products Ab, aB, AB and ab (Figure 2.7). The ordered tetrad of *Neurospora* is convenient because the products of the first division are located within one half of the ascus.

To set up strains for sexual reproduction and subsequent genetic analysis, the strain designated as the female is grown first, and conidia from the male are spread over the female culture. Perithecia develop within a few days, and acsi are mature and start to discharge ascospores by ten days. Spores collect on the side of the incubation tube, and can be taken for random spore analysis. Alter-

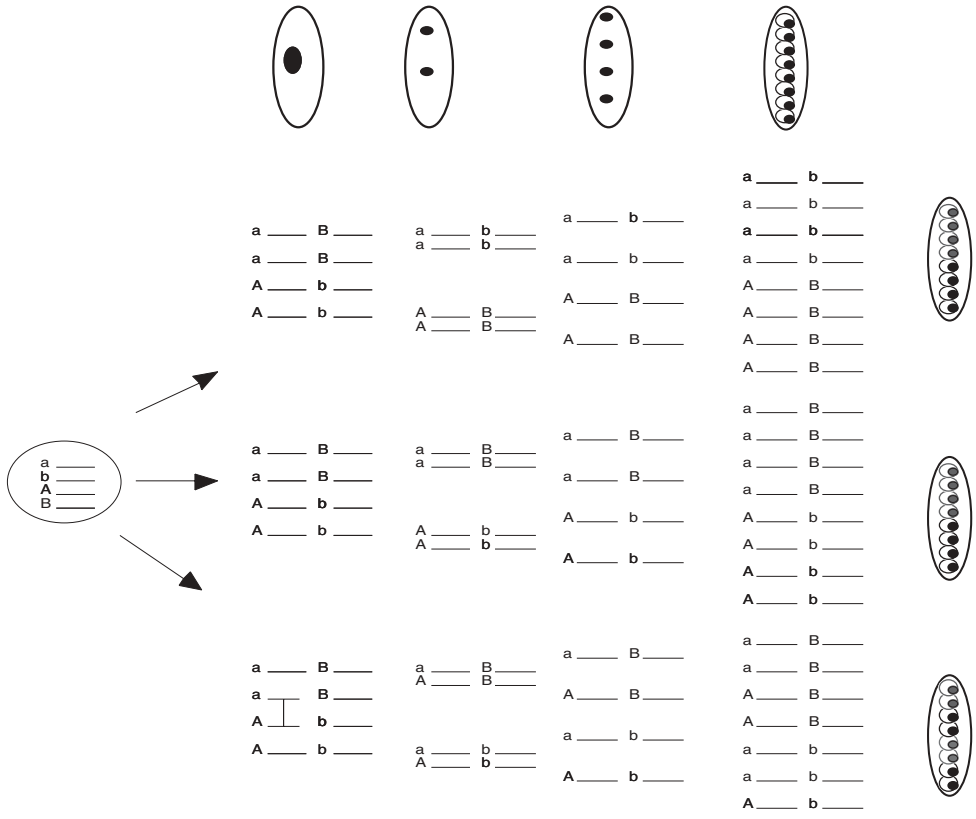

Figure 2.7 Chromosome assortment in *Neurospora crassa* (Adapted from Davis and De Serres, 1970)

natively, a perithecium is dissected out and crushed in a water droplet to release asci. An ascus is pressed with a needle to discharge a spore, break the ascus, and subsequently release the remaining spores. Spores are teased apart in order and separately analysed.

Prior to sequencing of the genomes, tetrad analysis was used for linkage analysis and mapping of genes. Reference strains with known markers were used in crosses with a test strain containing the gene of interest to detect any linkage (extent of recombination demonstrated by ascospore products), and to map the gene relative to the known position of the marker gene in the reference strain.

Aspergillus nidulans

Analysis of ascospores is different in *Aspergillus nidulans*, since it is not readily amenable to traditional tetrad analysis. However, Mendelian segregation for all

unlinked nuclear markers is demonstrated in random spore samples. Sexual reproduction for genetic analysis involves growing two strains of different auxotrophy and other genetically distinct markers, such as spore colour, on media that selects against the individual strains and thus forces heterokaryon formation. The plate containing the resulting heterokaryons is sealed to prevent any aeration, and after 2–3 weeks the fruiting bodies or cleistothecia form. Since the asci are extremely fragile, they are not dissected out from the cleistothecium, so traditional tetrad analysis is not usually performed. Instead, a cleistothecium is rolled onto a semi-solid water/agar surface to remove other cellular tissue, then crushed and spread across a plate to observe segregation of markers such as ascospore colour. Some cleistothecia are derived from self-fertilization, which is evident upon analysis of ascospore colours of the products. A stock of the spores from the crushed cleitothecium is maintained and tested on different selective media to determine the genotype of the individual products and extent of recombination.

2.5.2 Molecular methods for genetic screens

Transformation

S. cerevisiae was the first fungus to be transformed with exogenous DNA, and the development and refinement of this technology has led to the ability to effectively manipulate the genome of this yeast. Initial proof of the transformation potential of *S. cerevisiae* involved the construction of a yeast strain that contained two separate point mutants within a single locus, preventing any reversion of the gene to functionality, followed by introduction of a bacterial plasmid containing a cloned copy of the inactivated gene. A technique initially developed for protoplast (cells digested of their cell walls) fusion was modified to allow uptake of the DNA into the yeast cells, and proof that the exogenously added DNA was the source of the restored function of the missing gene was provided by the detection of the sequence of the bacterial plasmid in the clones that contained the restored function. There have been many modifications of the initial transformation protocol; currently, the use of electroporation or treatment of cells with lithium salts to trigger DNA uptake has essentially replaced the initial protocol of generating protoplasts, which had to regenerate their walls in an osmotically stabilized medium.

Transformation of filamentous fungi is performed either in protoplasts or in asexual conidia. In the former, protoplasts are typically transformed using PEG (polyethylene glycol) and calcium to facilitate entry of DNA. Conidia in some fungi, including *Aspergillus fumigatus*, can be transformed without degrading the cell wall, through electroporation. Lithium acetate-based transformation has also been used. Transformation efficiencies and optimum methods vary between fungi.

Plasmids, transforming DNA

Transformation requires that the DNA contains a selectable marker, which is normally a nutrition or drug-resistance gene. For example, if *pryG* encoding for orotidine-5′-phosphate decarboxylase, which is part of the uridine biosynthesis pathway in *Aspergillus nidulans*, is used as a marker on transforming DNA, the resulting positive transformants will grow on media lacking uridine and uracil, while untransformed cells will not. The use of such selectable markers is common to all fungal transformation systems.

The initial transformation of yeast cells was not highly efficient, and involved sequences that integrated into the genome. The great utility of episomal DNA sequences for the transformation of bacterial cells led to the search for equivalent tools for the manipulation of *S. cerevisiae*. These tools took two forms: episomes based on the backbone of the endogenous yeast 2μ plasmid, and episomes that contained origins of replication from the chromosomes. The 2μ plasmids contained sequences for efficient segregation, and thus were more stable than those based solely on the ARS (autonomous replication sequence) elements derived from chromosomes. However, a further introduction of centromeric sequences, which provide efficient segregation and maintain a low plasmid copy number, allow ARS-based plasmids to be very stable. Such plasmids can be maintained for many generations in the absence of selection, and now provide the tools for the molecular manipulation of yeast cells. The selection of the plasmids typically involves nutritional markers – the standard markers (*URA3*, *HIS3*, *TRP1* and *LEU2*) represent genes that were initially cloned by complementation of *E. coli* mutations, and were available in cloned form prior to the development of yeast transformation. Dominant drug-resistant markers are also available for selection of plasmids; resistance to G418 has been a useful marker in *S. cerevisiae*. Essentially all commonly used yeast transformation plasmids include selection markers and replication origins for propagation of the plasmid in *E. coli*, so the plasmids can be shuttled between the prokaryotic and eukaryotic hosts.

Most filamentous fungi lack the ability to maintain extrachromosomal plasmids, in contrast to the yeast *Saccharomyces cerevisiae*, so transformed DNA typically integrates homologously or heterologously in the genome. The frequency of homologous recombination increases with increasing length of homologous DNA. Transforming DNA therefore typically involves a vector background containing an *E. coli* origin of replication and ampicillin-resistance marker to allow replication and selection in bacteria for plasmid propagation, as well as a fungal-specific marker and the desired gene sequence.

Transformation is performed to either knock out a gene, replace a gene with a mutated version or control gene expression. A gene knockout construct contains a marker gene surrounded by 5′ and 3′ flanking DNA of the gene of interest, so that the linear ends can recombine with the endogenous 3′ and 5′ flanking ends of the endogenous gene, allowing its replacement with the marker

gene. Gene expression can be controlled by recombining a regulatable promotor in front of the endogenous ORF. In *Saccharomyces cerevisiae,* the promotor for galactose is often used, where galactose and glucose in the medium regulate overexpression and repression, respectively. In *Aspergillus nidulans,* the *alcA* promotor is commonly used, and becomes overexpressed if the cells are grown on media containing ethanol as a carbon source, or repressed when cells are grown on media containing glucose.

Genetic screens

In a post-genomic fungal world where genomes are sequenced and annotated, and genes no longer need to be mapped to chromosomes through traditional genetic techniques, genetic analysis is still a very powerful tool, particularly in construction of mutant screens. Genetic screens in filamentous fungi have uncovered an enormous amount of information about diverse cellular processes, and in many cases identified the first examples of conserved genes and their functions. Pioneering work in *Neurospora crassa* by Beadle and Tatum demonstrated that individual genes encoded for individual enzymes, bringing together genetic and biochemical analyses for the first time. Subsequent screens in this organism have provided novel information on gene conversion, recombination, circadian rhythms, gene silencing and DNA methylation. Genetic screens in *Aspergillus nidulans* and *Saccharomyces cerevisiae* continue to uncover novel information in diverse areas including cell cycle regulation, cellular motors and cytoskeletal dynamics, signalling and development.

Classical screening

Classical screening involves mutagenizing cells, typically with UV light, radiation or chemicals, then allowing growth of survivors. This approach was used by Beadle and Tatum in 1941 to uncover the metabolic mutants in *Neurospora crassa* (Figure 2.8). Single conidia colonies were exposed to X-irradiation on complex medium (arrow in Figure 2.8), and transferred to minimal medium. Growth on complex versus minimal medium was screened, and any colony that could not grow on minimal medium was considered to contain a nutritional mutation. The strain was maintained on complex media but subsequently tested for the restoration of growth on minimal media containing defined additives, such as tyrosine, leucine or alanine. If growth was restored only when tyrosine was added, for example (see Figure 2.8), the specific mutation could then be identified. From this approach, strains containing mutations in vitamin B_6, vitamin B_{12} and *para*-aminobenzoic acid were uncovered. The mutant strains were crossed with wild-type parental strains to ensure that only a single gene was mutated.

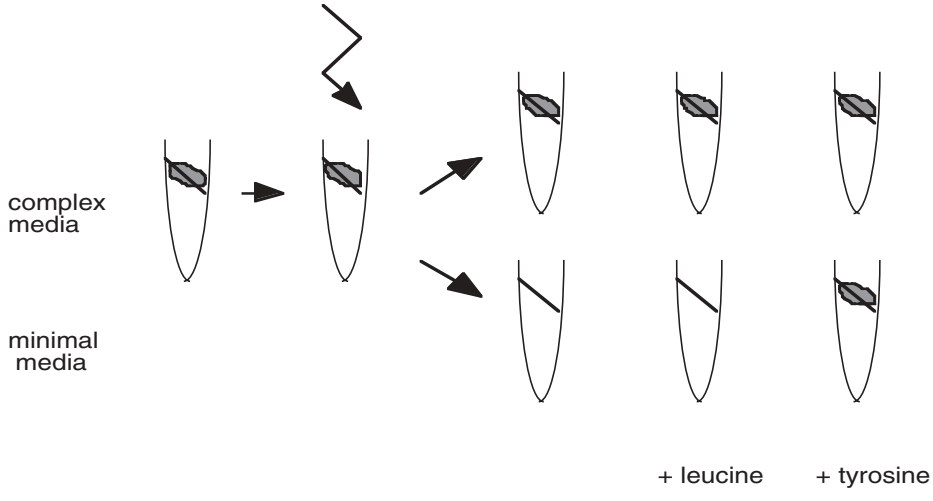

Figure 2.8 Genetic screen identifying metabolic mutants in *N. crassa*

Another classical mutagenesis screen identified many genes that control a circadian rhythm *in Neurospora crassa*. Circadian rhythms are present from fungi to humans, and are biological processes that are sensitive to light and temperature and therefore oscillate every 24 h in the absence of environmental signals. The timing of conidia formation in *Neurospora crassa* is regulated by such an internal clock, and genes involved in responding to the clock were uncovered by using mutagenesis and race tube assays. When inoculated at one end of a long glass horizontal tube, called a race tube, *Neurospora* hyphae grow to the other end, creating a periodic banding pattern along the tube from the coloured conidia that form every 24 h. Mutagenized conidia were placed at one end of the tube, and changes in banding pattern, reflecting changes in day length relative to control strains, were screened. The *frequency, period* and *chrono* genes were uncovered, most of which have homologues involved in clock functions in *Drosophila melanogaster* and humans.

In *S. cerevisiae*, classical screens were applied to identify the cell division cycle (*cdc*) genes that uncovered many of the key controlling elements underlying what is now considered the universal eukaryotic cell cycle. Because defects in the cell cycle blocked cellular proliferation, it was necessary to identify conditional mutations, in this case temperature-sensitive mutants that arrested with a uniform terminal phenotype. In general, the uniform terminal phenotype arose because a particular cellular function necessary for completion of the cell cycle was missing at the restrictive temperature. Key proteins identified through this screening included Cdc28p, the cyclin-dependent protein kinase controlling both mitosis and the G1 to S transition, Cdc35p, which is adenylyl cyclase, Cdc12p, which is a septin, and Cdc9p, which is DNA ligase. Novel cellular processes

were uncovered through the analyses of these mutations, and genes identified initially as *cdc* mutants form the underpinning of much of the current cell biology of *S. cerevisiae*.

Other strategies for gene identification in *S. cerevisiae* involved modifications of the classical screen approach that added enrichment protocols to improve the frequency of mutant identification. For example, screening for mutations that blocked the process of secretion was made more efficient by treating the mutagenized cell population to a density enrichment prior to looking for temperature-sensitive mutants.

Selected screens

The traditional screen for mutants has become more powerful and specific through introduction of various types of selection. A classic screen designed to identify mutants of the cell cycle in *Aspergillus nidulans* was designed by Ron Morris in 1975 (Figure 2.9(a)). Conidia were mutagenized with UV light (arrow in Figure 2.9(a)), then allowed to grow at 32 °C. Cells were replica spotted onto medium at 42 °C to screen for temperature sensitivity. Cells that could not grow

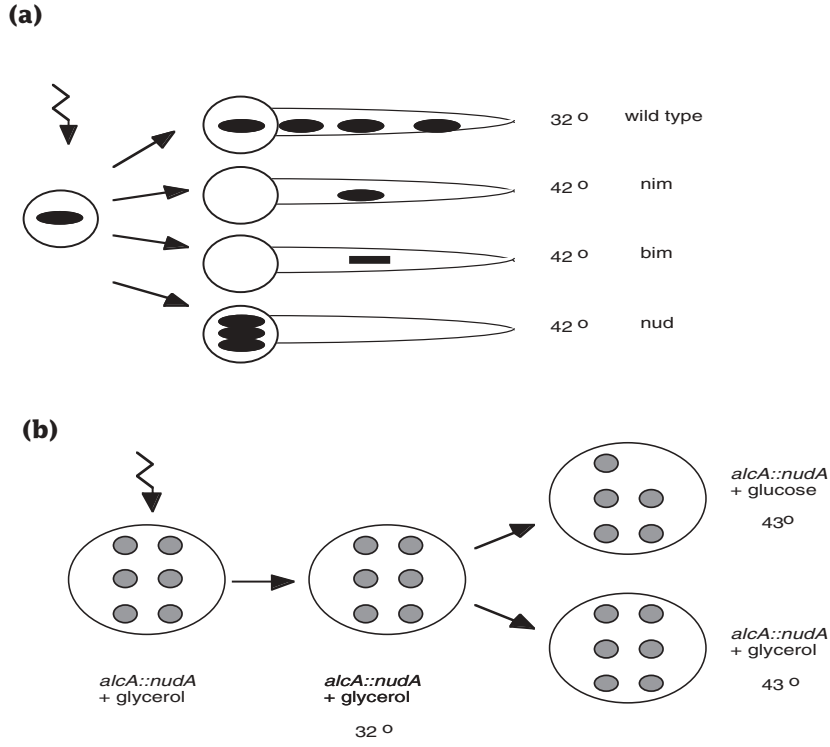

Figure 2.9 Selected genetic screens identifying cell cycle mutants in *A. nidulans* (Adapted from Casselton and Zolan, 2002)

at the restrictive temperature were analysed for phenotype, and stained to visualize nuclei. Several classes of mutants were uncovered, and characterized as the *nim* (never-in-mitosis), *bim* (blocked-in-mitosis) and *nud* (nuclear distribution) mutants. These acronyms have subsequently been used for mutants in other systems, including *Schizosacchromyces pombe* and *Saccharomyces cerevisiae*. The genes responsible for the mutant phenotypes were identified through complementation analysis. Several of the genes, including NIMA kinase, which is essential for the G2/M transition, and BIMC, which is a kinesin motor, were the founding members of families of related proteins subsequently found in other organisms, from yeast to man. Another gene identified in the screen, *nudF*, has a homologue in humans that, when mutated, is the cause of the human neuronal disease lissencephaly.

In other landmark genetic screens performed in *Aspergillus nidulans*, resistance to the anti-microtubule drug benomyl was incorporated to select for mutations in tubulin. The subsequent *benA* mutants were confirmed to be β1- and β2-tubulin. Suppression analysis of the *benA* mutants was then used to identify mutations that affected proteins that interacted with β-tubulin. Based on work done in bacteriophage, if mutations of a protein prevented its interaction with another protein and thereby inactivated its function, a compensatory mutation in the second factor, which restores the ability of the two proteins to interact, would also restore function. The *benA* mutant was therefore subjected to another round of mutagenesis, and temperature-insensitive revertants were isolated. Through subsequent analysis, α-tubulin was identified, which forms a dimer with β-tubulin. Suppression screening of the *benA* mutant also identified the *mipA* mutants, which upon mapping and cloning provided the first example of γ-tubulin in any organism.

A classic selection approach was applied to identify mutants defective in the mating process in *S. cerevisiae*. This selection was based on the observation that a functioning mating response pathway resulted in cells arresting the cell cycle in the presence of the mating pheromone produced by cells of the opposite cell type. Thus mutagenized *MAT**a*** cells were treated with the mating pheromone α-factor, and the only cells that could grow to form colonies would be those that were insensitive to the pheromone. This selection allowed the identification of many of the key kinases and regulators of this pathway.

The mutagenesis screen has more recently been adapted to identify synthetic lethals, which can also uncover potential functionally interacting proteins. In one example, a strain of *Aspergillus nidulans* in which *nudA* (dynein) was placed under control of the *alcA* regulatable promotor was utilized (Figure 2.9(b)). On glucose, dynein expression was turned off, but on glycerol, dynein was expressed. After UV mutagenesis (arrow in Figure 2.9(b)), the strain was grown on glycerol at 32 °C, then replica spotted onto glycerol or glucose at the restrictive temperature of 42 °C. The ability of temperature-sensitive mutations to grow on glycerol (+dynein) but not on glucose (–dynein) was screened. The resulting strains were back-crossed to wild type and *nudA* mutant strains to

isolate single and double mutants. The mutants, called *sld* for synthetic lethal with dynein, were cloned and determined to be homologues of Bub1p and Bub3p spindle checkpoint factors.

Insertional mutagenesis

Insertional mutagenesis is highly advanced in *S. cerevisiae*. Initially circular plasmids containing selectable markers were transformed into cells and were found to integrate at homologous sites quite efficiently. Markers inserted into repetitive sequences such as the ribosomal DNA allowed the analysis of both mitotic and meiotic recombination. The recognition that double-stranded breaks both dramatically enhanced the frequency of insertion, and provided for efficient targeting to the homologous site in the genome, greatly improved the technology of directed gene replacement. Several strategies are currently available for disrupting gene function by replacing part or all of a gene with foreign DNA, thus inactivating the function of the gene of interest. The use of such homology-driven gene inactivation has been extensively applied to investigations of individual genes. A modification of this approach has been used to provide efficient insertional mutations for random yeast sequences. In this approach, a library of random yeast DNA inserts in the 10 kB range is constructed in an *E. coli* plasmid vector. This library is transformed into *E. coli*, and the *E. coli* strain is then subjected to a transposon mutagenesis with a modified bacterial transposon containing at least a selectable marker for *S. cerevisiae*. Plasmids are selected that have picked up a transposon insertion, and many of these insertions have occurred in the yeast DNA portion of the plasmid. The yeast inserts are liberated from the vector by restriction digest, and the digestion products used to transform yeast cells. Selection for the yeast marker on the transposon allows detection of integration events, and these integration events represent transposon insertions into essentially random regions of the yeast genome – the overall distribution dependent on the randomness of the initial library of yeast sequences and the randomness of the transposon insertions into this library. Strategies that made use of the endogenous yeast transposable element to do the transposon hops directly in yeast have also been developed, but were limited by the non-random characteristics of the insertion locations.

Insertional mutagenesis has also been applied to genetic screens in filamentous fungi. In REMI (restriction-enzyme-mediated insertions), a plasmid that has been linearized is transformed into protoplasts in the presence of the restriction enzyme that was used for cutting. The enzyme is thought to cleave the genomic DNA at restriction sites that are compatible with the ends of the cut plasmid, allowing integration at multiple sites throughout the genome, and the creation of multiple, potentially mutagenized transformants. To determine where the plasmid integrated, and the identity of the mutagenized gene, genomic DNA from the strain is isolated and cut with enzymes that would not cut within the plasmid itself. Fragments of DNA that contain the plasmid plus flanking

genomic DNA are allowed to ligate, then transformed into *E. coli* to rescue the plasmid using a plasmid-specific drug-resistance marker.

A more efficient system for random mutagenesis incorporates a tag on the mutagenic DNA, as is the case with the transposon mutagenesis library used in *Saccharomyces*. In the system termed TAGKO, a cosmid library containing the genome of *Magneporthe griseae* was mutagenized with a transposon. The individual mutated cosmids were sequenced off the transposon and annotated. The cosmids with known mutated genes were then transformed into the fungus to determine the effect of replacing the endogenous gene with the mutated version.

2.6 Conclusions

Fungi have important interactions with humans. Many of these organisms are economically significant, such as the bakers' or brewers' yeast *Saccharomyces cerevisiae* generating our bread and alcohol, or medically important, such as the human pathogens *Candida albicans* and *Aspergillus fumigatus*. They represent, as well, experimental systems that have been extensively exploited to investigate the molecular details of eukaryotic cell function. In particular, the fungi include some of the most highly developed eukaryotic genetic systems. From early work in *Neurospora* leading to recognition of the relationship of genes and enzymatic functions, to more recent work in the yeasts *S. cerevisiae* and *S. pombe* that has uncovered the molecular basis of control of cell proliferation, we can see that the isolation and characterization of genetic variants of fungal cells has revolutionized our understanding of cellular function.

A key characteristic of genetic systems is that they can be exploited to create new combinations of variations. This ability is highly developed in fungal systems. The capacity to identify all the products of the meiotic event has allowed for such a sophisticated analysis of meiotic recombination that fungal systems have played leading roles in the development of molecular models of DNA recombination. Only though the analysis of complete individual meioses would we be able to detect the gene conversions and post-meiotic segregations that established the existence of hetroduplex DNA during the recombination process.

We have only touched the surface of the uses of fungi in uncovering details of the function of eukaryotic cells. Our current work focuses on a few fungi, but the ability to sequence whole genomes promises to allow many more organisms to be investigated through the tools of genetics and genomics. In the future we should be able to study the characteristics of more and more of the fungal diversity, and investigate the current model organisms with even greater sophistication. Based on the success of the investigations of the prior decades, we can expect the fungi to continue to be in the forefront of research in providing new information on the diversity of cellular processes in the future.

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2.8 Revision questions

Q 2.1 What are some advantages of using fungi as model organisms to investigate biological questions?

Q 2.2 Describe some key structural differences between yeast and filamentous fungi.

Q 2.3 What are some differences in ascus structure between *S. cerevisiae* and *N. crassa,* and what are the advantages of some of these features for genetic analysis?

Q 2.4 Describe the first genetic screen used in fungi, and the main findings of the study.

Q 2.5 Describe different selection criteria used in devising genetic screens in fungi. Give examples.

Q 2.6 Genetic screens have not been extensively used in the analysis of *C. albicans*. Why?

Q 2.7 In *S. cerevisiae*, the genes *NUP60* and *TRP1* are located close to centromeres of chromosomes 1 and 4 respectively. A strain with a mutant *TRP1* locus (*trp1*) and a functional *NUP60* locus (*NUP60*) has the genotype *trp1 NUP60*. This strain was crossed to a strain with a functional *TRP1* gene and a mutant *NUP60* gene (*TRP1 nup60*). The segregation of these two markers was examined in 20 dissected tetrads. The patterns of spores could be parental ditypes (PD) where the four spores consist of two *trp1 NUP60* and two *TRP1 nup60* spores, nonparental ditypes (NPD) where the four spores consist of two *trp1 nup60* spores and two *TRP1 NUP60* spores, or tetratypes (TT) where all four spore combinations (*trp1 nup60, trp1 NUP60, TRP1 nup60*, *TRP1 NUP60*) are present. In these 20 tetrads, would tetratype tetrads be rare or common? Would this change if the starting parents were the double mutant *trp1 nup60* and the wild-type *TRP1 NUP60*?

Q 2.8 What are some key differences in the mating loci of *N. crassa* and *C. cinereus* compared to that of *S. cerevisiae*?

Q 2.9 What is parasexual genetic analysis, where has it been used and what information can it provide?

Q 2.10 What is a major difference between transformation plasmids used in *S. cerevisiae* and those used in most filamentous fungi, and how is this difference advantageous for genetic screens?

3

Fungal Genetics: A Post-Genomic Perspective

Brendan Curran and Virginia Bugeja

3.1 Introduction

Ushered in by the exponential accumulation of DNA sequences in databases throughout the world, the post-genome era is characterized by the application of computer technology to a deluge of information arising from the large-scale parallel analysis of biological molecules. With information accumulating from an ever-increasing network of resources, the challenge now is to re-integrate these molecular details to reveal the secrets of the dynamic processes they mediate within the cell: an experimental and theoretical approach referred to as systems biology.

3.1.1 The yeast *S. cerevisiae* – a cornerstone of post-genomic research

A forerunner of the much more ambitious project to sequence the human genome, the yeast *Saccharomyces cerevisiae* entered the history books in 1996 as the first eukaryotic organism to have its entire genome sequence deposited in a computer database. With other eukaryotic genomes entering the databases, and the development of computational tools for capturing, storing, displaying, distributing and comparatively analysing the rapidly accumulating information, biologists were able for the first time to analyse and compare entire eukaryotic genomes – the post-genomic era had begun. However, the DNA sequence of *S. cerevisiae* was just the starting point for large-scale molecular analysis of eukaryotic cells. Within a

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very few years this extremely tractable model organism rapidly yielded a whole series of molecular secrets on a global scale: each of its genes was systematically deleted in search of phenotypes; technology to allow its global mRNA profiles to be identified was developed; all possible protein–protein interactions were examined; and its cellular metabolites exhaustively characterized. In short, this simple eukaryote became the key to post-genomic research.

3.1.2 Of *-omics* and systems biology

Whereas pre-genomic research was characterized by hypothesis-driven sequential experiments, post-genomic research is driven by the massively parallel analysis of biological information, followed by pattern recognition within datasets. *Genomics*, the accumulating and analysis of massive amounts of DNA sequence data, was possible because an appropriate technology platform was put in place. The components of this 'platform' included automated preparation of DNA, automated sequencing of multiple DNA fragments and the computational tools to store and then process the generated data. As the science of examining genomes is referred to as genomics, so too complementary global analyses of other biological molecules are given -omic suffixes, namely *transcriptomics*, which is concerned with the accumulation of information on RNA sequences and their expressed levels; *proteomics* deals with protein sequences, protein structures, protein levels and protein interactions both with DNA and other proteins. Each one of these has required the development of automated and computationally intensive technology platforms to complement the high-throughput DNA sequence and analysis platform, which characterizes genomics. These include *DNA array technology*, which allows multiple DNA or RNA sequences to be simultaneously identified, and *mass spectrometry* for the identification of multiple protein samples. Once again the yeast *S. cerevisiae* has led the way in the development of these platform technologies. Much more than this, however, *S. cerevisiae* has now become a central player in the development of an entirely new approach to biological research – *systems biology*. This newly emerging field uses a cross-disciplinary approach involving biology, chemistry, physics, mathematics, computer science and engineering to develop working models of how these molecules interact to generate biological phenomena.

3.2 Genomics

3.2.1 Analysing encoded information

Genomics, the study of whole genomes, encompasses (1) searches for patterns of relatedness within and between genome sequences and (2) attempts to ascribe specific biological functions to particular DNA sequences within those genomes.

The successful completion of fungal genomes, in particular the annotated genomes of the three model organisms *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Neurospora crassa*, has excited much interest within and beyond the fungal community. These, and 24 other genome-sequencing projects at various levels of completeness, can be accessed at http://www.ncbi.nlm.nih.gov/genomes/FUNGI/funtab.html. Fungal sequencing projects to date include model organisms, fungi with relevance to healthcare and fungi of agricultural and commercial importance (Table 3.1). In addition, a worldwide group of fungal biologists constituting the Fungal Genome Initiative have produced a 'white paper' cogently arguing the case for the sequencing, for comparative purposes, of a further 25 or so fungal genomes. Comparative analysis of fungal genomes is set to revolutionize our understanding of this ancient and evolutionarily diverse group of organisms. Moreover, given the underlying unity of biological information, many of these findings will also be relevant to our understanding of plant and animal biology.

3.2.2 Pattern recognition within and between genome sequences

Without ever revealing anything specific about biological function, computer analysis of a complete genome sequence provides many interesting and revealing facts about its topology and evolutionary history. For instance, computer programs use pattern recognition to identify tracts of DNA that start with a methionine codon and run for another 99 codons, without hitting a nonsense one (Figure 3.1). Such sequences, referred to as open reading frames (ORFs), are then annotated as highly likely to encode proteins. More advanced programs even allow for the presence of introns by being able to recognize intron–exon boundaries. Sequence recognition programs provide a detailed map of the genome and much useful information including the number of genes, the size of the gene sequences, the presence or absence of introns, the spacing of genes within the chromosomes, and much more besides.

Such programs identified the precise position of approximately 6000 genes in the *S. cerevisiae* genome – the best labours of yeast geneticists during the preceding 40 years had revealed the positions of fewer than 1000 genes! Sequence analysis also revealed that the yeast genome is extremely compact, with 70 per cent consisting of protein-encoding sequences – protein-encoding sequences that are almost completely devoid of introns. In addition, the number, and chromosomal locations, of genes encoding non-coding RNA species such as ribosomal RNA, small nuclear RNAs and transfer RNA (tRNA) were also identified. A similar pattern-recognition analysis revealed that the *S. pombe* genome carries 600 fewer protein-encoding genes (approximately 4900 in all), whereas a multicellular filamentous cousin, *Neurospora crassa*, requires twice that number: 10 000 genes. Unlike *S. cerevisiae*, the protein-encoding genes in both *S. pombe*

Table 3.1 Fungal sequencing projects to date

Organism	Genome sequencing status	Significance	Useful URLs
Saccharomyces cerevisiae	Completed 1996	Biotechnology, model organism	http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=4932 http://www.yeastgenome.org/
Schisosaccharomyces pombe	Completed 2002	Model organism	http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=4896
Neurospora crassa	Completed 2003	Model organism	http://www-genome.wi.mit.edu/
Aspergillus fumigatus	Completed 2004	Medical – causative agent of aspergillosis	http://www.sanger.ac.uk/Projects/A_fumigatus/
Candida albicans	Completed 2004	Medical – most common human pathogen	http://sequence-www.stanford.edu/group/candida/index.html http://www.sanger.ac.uk/Projects/C_albicans/
Cryptococcus neoformans	Completed 2001	Medical – leading cause of infectious meningitis	http://www-sequence.stanford.edu/group/C.neoformans/index.html
Ustilago maydis	Completed 2003	Agricultural – fungal pathogen causing corn smut disease in cereal crops	http://www.broad.mit.edu/annotation/fungi/ustilago_maydis/index.html
Magnaporthe grisea	Completed 2003	Agricultural – the causal agent of rice blast disease	http://www.broad.mit.edu/annotation/fungi/magnaporthe/background.html http://www.ncbi.nlm.nih.gov/mapview/map00.cgi?taxid=148305

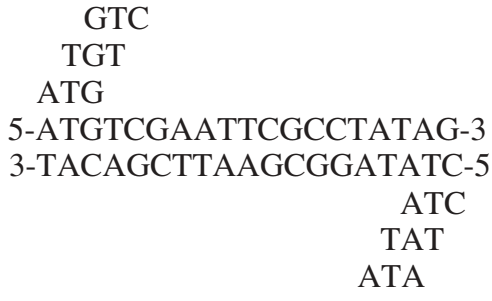

Figure 3.1 Finding an ORF. Finding the ATG methionine codon requires a computer search of six different reading frames. An ORF continues for at least a further 99 codons before hitting a nonsense codon

Table 3.2 Genome topology of model fungi

Fungal species	Organismal complexity	Genome size	Protein encoding sequences	Gene density (excluding introns)
S. cerevisiae	Budding yeast	12.07 Mb	5 500	1 every 2.09 kB
S. pombe	Fission yeast	13.8 Mb	4 900	1 every 2.53 kB
N. crassa	Filamentous fungus	41 Mb	12 000	1 every 3.1 kB

and *N. crassa* carry introns. The percentages of the genome consisting of protein-encoding sequences are also lower in both than in *S. cerevisiae*. Finally, the gene density of the latter two is also significantly lower than that found in their cousin (Table 3.2).

Having identified the majority of the ORFs in a genome, a computer-aided comparison that aligns each ORF against all other ORFs within the same genome can reveal much about the evolutionary history of a genome. One such study revealed that an ancient genome duplication occurred during the evolutionary history of *S. cerevisiae*. ORF comparisons within the genome sequence identified 55 segments of chromosomes carrying three or more pairs of homologous genes – despite this being a haploid genome. This supported a model in which two ancestral diploid yeast cells, each containing about 5000 genes, fused to form a tetraploid. Most of the duplicate copies were then subsequently lost by deletion as this species evolved, leaving it in its haploid phase with approximately 5500 genes. It is estimated that protein pairs derived from this duplication event make up 13 per cent of all yeast proteins. The same study also

Table 3.3 Evolutionary history of model fungal genome

Fungal species	Evidence of former genome duplication	Evidence of gene duplication	Evidence of transposon activity
S. cerevisiae	Yes	Yes	Abundant
S. pombe	No	Yes	Abundant
N. crassa	No	No	Very little

revealed that transposable elements play an important role within the genome: there are 59 such elements, constituting 2.4 per cent of the entire genome. On the other hand, *intragenomic* searches in the *S. pombe* genome sequence failed to find evidence of large-scale *genome duplications*. However, they did find evidence suggesting that *gene duplication* played a key role in the evolution of this yeast. In fact, as many as 10 per cent of the ORFs can be defined as having paralogous sequences within this genome (paralogous genes being homologous genes that arose by gene duplication within the same species). ORF comparisons also reveal that transposable elements are important to this yeast; there are 11 intact transposable elements, which account for 0.35 per cent of the genome (Table 3.3).

Within-genome comparison of ORFs in *N. crassa*, the multicellular distant cousin to both of these yeasts, fails to find evidence for large-scale genome duplication, gene duplication or transposon activity. The level of redundancy among ORFs of *N. crassa* is very low; fewer than 4 per cent can be defined as having a paralogous ORF within the genome. This analysis suggests that the genome did not undergo duplication, that gene duplication is rare and that transposon activity is extremely limited (Table 3.3). The paucity of transposable elements and paralogues in the *Neurospora* genome can perhaps be explained by the process of RIP (repeat-induced point mutation), which involves the hypermutation of duplicated sequences of more than 1 kb in length during sexual development in this fungus. However, these results pose this interesting biological question: can *Neurospora* currently utilize gene duplication as a means of gene diversification?

3.2.3 Assigning biological functions to fungal genome sequences

Although analyses of individual genome sequence databases can be interesting and informative in their own right, biologists are more interested in using the sequence to understand the biology of the cell. *Functional genomics*, the name

given to this process of *assigning functions to ORF sequences*, is multifaceted. Potential functions can be attributed to some ORFs by identifying similar sequences of known function in existing databases (*in silico analysis*). Alternatively ORFs of unknown function can be disrupted in order to produce a phenotype (*reverse genetics*). Failing this, clues to the potential biological significance of unknown ORFs can be gleaned by large-scale comparisons of genomes from a variety of different organisms. This so-called *comparative genomics* can identify potential gene functions by virtue of the fact that certain sequences are present in some genomes but absent from others (*identification by association*).

3.2.4 Identification by *in silico* analysis

Even before genome-sequencing projects were initiated research groups around the world had already set up databases containing large numbers of annotated DNA, RNA and protein sequences. As these grew exponentially, computational techniques were developed to make these resources readily available and searchable. These databases, which frequently annotate DNA sequences with encoded protein functions, provided invaluable information on the potential or actual function of proteins encoded by newly identified ORFs. Blast searches of these resources were used to annotate about 50 per cent of the ORFs in the *S. cerevisiae* genome. This allowed biologists to discover the percentage of genes that this simple eukaryotic cell dedicated to various aspects of cellular biology: there are in excess of 600 metabolic proteins, in excess of 400 proteins involved in intracellular trafficking or protein targeting and approximately 200 transcription factors. Specific genes whose existence in *S. cerevisiae* had hitherto been in doubt were also identified in this way: histone H1 was found on chromosome XVI (Figure 3.2), and a yeast γ-tubulin gene, which had previously eluded yeast geneticists despite intensive efforts, was identified on chromosome XII.

3.2.5 Identification by reverse genetics

Although *in silico* analysis is useful, it can only annotate ORFs from new genome sequences using functions that had previously been identified elsewhere. Indeed, because it frequently identifies protein domains (rather than the entire protein sequence), the annotation is often no more than a general assignment (e.g. ORF 'X' encodes a 'protein kinase' or ORF 'Y' encodes a 'transcription factor'). A specific and unequivocal assignment is possible, however, if a phenotype can be identified when a particular ORF is deleted or disrupted. It was for this reason that an international consortium of scientists undertook the daunting task of using *S. cerevisiae*'s ability to undergo homologous recombination to produce yeast strains in which specific ORFs had been deleted. It was hoped that such specific deletions would provide insight as to the biological

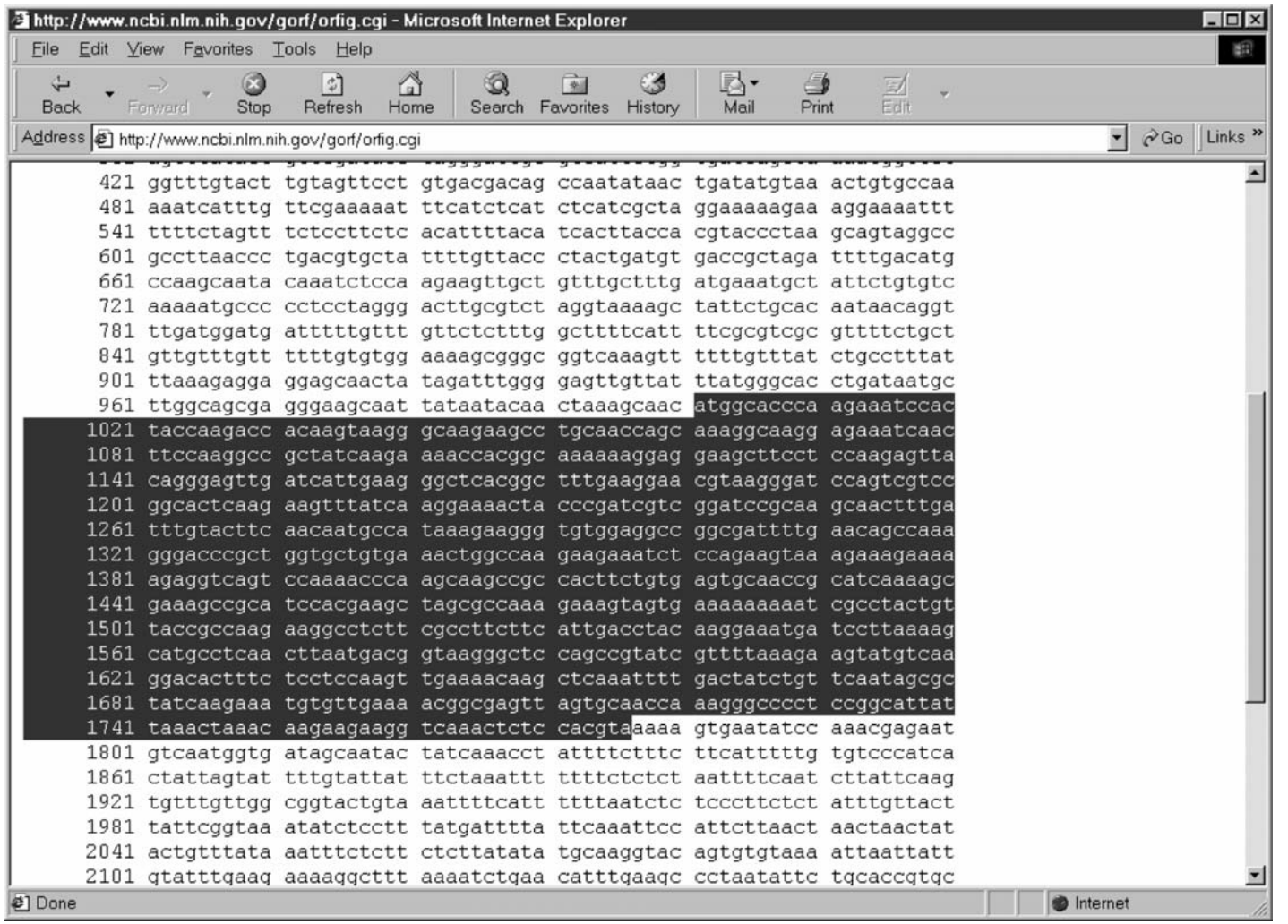
http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi - Microsoft Internet Explorer
File Edit View Favorites Tools Help
Back Forward Stop Refresh Home Search Favorites History Mail Print Edit
Address http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi
Go Links
421 ggtttgtact tgtagttcct gtgacgacag ccaatataac tgatatgtaa actgtgccaa
481 aaatcatttg ttcgaaaaat ttcatctcat ctcatcgcta ggaaaaagaa aggaaaattt
541 ttttctagtt tctccttctc acatttttaca tcacttacca cgtaccctaa gcagtaggcc
601 gccttaaccc tgacgtgcta ttttgttacc ctactgatgt gaccgctaga ttttgacatg
661 ccaagcaata caaatctcca agaagttgct gtttgctttg atgaaatgct attctgtgtc
721 aaaaatgccc cctcctaggg acttgcgtct aggtaaaagc tattctgcac aataacaggt
781 ttgatggatg atttttgttt gttctctttg gcttttcatt ttcgcgtcgc gttttctgct
841 gttgtttgtt ttttgtgtgg aaaagcgggc ggtcaaagtt ttttgtttat ctgcctttat
901 ttaaagagga ggagcaacta tagatttggg gagttgttat ttatgggcac ctgataatgc
961 ttggcagcga gggaagcaat tataatacaa ctaaagcaac atggcaccca agaaatccac
1021 taccaagacc acaagtaagg gcaagaagcc tgcaaccagc aaaggcaagg agaaatcaac
1081 ttccaaggcc gctatcaaga aaaccacggc aaaaaaggag gaagcttcct ccaagagtta
1141 cagggagttg atcattgaag ggctcacggc tttgaaggaa cgtaagggat ccagtcgtcc
1201 ggcactcaag aagtttatca aggaaaacta cccgatcgtc ggatccgcaa gcaactttga
1261 tttgtacttc aacaatgcca taaagaaggg tgtggaggcc ggcgattttg aacagccaaa
1321 gggacccgct ggtgctgtga aactggccaa gaagaaatct ccagaagtaa agaaagaaaa
1381 agaggtcagt ccaaaaccca agcaagccgc cacttctgtg agtgcaaccg catcaaaagc
1441 gaaagccgca tccacgaagc tagcgccaaa gaaagtagtg aaaaaaaaat cgcctactgt
1501 taccgccaag aaggcctctt cgccttcttc attgacctac aaggaaatga tccttaaaag
1561 catgcctcaa cttaatgacg gtaagggctc cagccgtatc gttttaaaga agtatgtcaa
1621 ggacactttc tcctccaagt tgaaaacaag ctcaaatttt gactatctgt tcaatagcgc
1681 tatcaagaaa tgtgttgaaa acggcgagtt agtgcaacca aagggcccct ccggcattat
1741 taaactaaac aagaagaagg tcaaactctc cacgtaaaaa gtgaatatcc aaacgagaat
1801 gtcaatggtg atagcaatac tatcaaacct attttctttc ttcatttttg tgtcccatca
1861 ctattagtat tttgtattat ttctaaattt ttttctctct aattttcaat cttattcaag
1921 tgtttgttgg cggtactgta aattttcatt ttttaatctc tcccttctct atttgttact
1981 tattcggtaa atatctcctt tatgatttta ttcaaattcc attcttaact aactaactat
2041 actgtttata aatttctctt ctcttatata tgcaaggtac agtgtgtaaa attaattatt
2101 gtatttgaag aaaagggctt aaaatctgaa catttgaagc cctaatattc tgcaccgtgc
Done
Internet

Figure 3.2 The sequence of the H1 gene in the *S. cerevisiae* database. Using an appropriate search, the bases encoding the H1 gene (blocked in above) were found as part of the sequence of chromosome 16

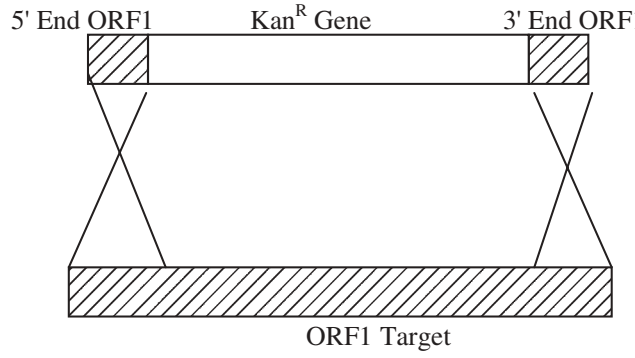

Figure 3.3 ORF knockout in the yeast *S. cerevisiae*. Appropriate PCR primers are used to generate hybrid DNA molecules consisting of the Kan^R selectable marker flanked by blocks of DNA consisting of the 5′ and 3′ ends of the ORF of interest. Homologous recombination in transformants knocks out the ORF of interest

function of the affected ORF. The EUROFAN project deleted and grossly characterized the mutant phenotypes from 758 ORFs of unknown function. A number of other European laboratories complemented this by undertaking a more focused range of deletion mutants. However, a transatlantic consortium undertook the most ambitious project: they used PCR to generate hybrid DNA molecules consisting of a selectable marker flanked by the 5′ and 3′ ends of the ORF of interest. They then transformed this into wild-type cells and selected for recombinants that had integrated the PCR fragment into the target ORF (Figure 3.3). By repeating this with each ORF in turn, they systematically deleted all 6000 or so genes. A number of mutant yeast libraries have also been generated using transposons to randomly generate mutations by insertional mutagenesis. Many of these strains have subsequently been characterized for phenotypes. Of the approximately 3000 ORFs of unknown function after the yeast sequence was released in 1996, such approaches have to date identified biochemical or physiological functions for more than 1000 of them.

Gene knockout technologies using a variety of molecular approaches are also valuable in the study of other fungi. With appropriate selectable markers and transformation procedures available for many pathogenic fungi including *Candida albicans*, *Candida dubliniensis*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Histoplasma capsulatum* and *Coccidioides immitis*, the application of gene knockout technology to investigate the basis of their pathogenicity is awaited with great interest. Such practical application of the basic approach taken in the investigation of *S. cerevisiae* will empower the development of antimycotic drugs in post-genomic research in these organisms. High-frequency homologous recombination is possible in a number of these, and complete or partial genome sequences for a number of pathogens are already available. However, the relationship between gene knockouts and pathogenicity is complicated by a number of factors including: the fact that high-frequency homol-

ogous recombination is not possible in all cases; different isolates (and the same isolate under different nutrient conditions) vary in their virulence; and in the case of *Candida* (a diploid), knockouts require two independent deletion events. Nevertheless, the application of reverse genetics to some of these organisms is already underway.

However, as it is not feasible to analyse all fungi in this way, alternative strategies are being devised. One such strategy outlined in the FGI white paper uses a process of 'guilt by association' to link ORFs of unknown function (so-called orphans) with biological function.

3.2.6 Identification by association

When the fission yeast *Schizosaccharomyces pombe* entered the history books as the second completed fungal genome, it became the sixth eukaryotic genome to enter the fully annotated genome databases. It also presented the first opportunity to undertake a comparative genomic analysis of fungal organisms, viz. *S. cerevisiae* and *S. pombe* – both to one another and also to other genomes. Using the genome sequence of *C. elegans* as a simple *multicellular* eukaryote, this analysis revealed that 681 ORFs (14 per cent) were uniquely present in *S. pombe*, 769 (16 per cent) were homologous to *S. cerevisiae* ORFs, and about two-thirds of the *S. pombe* ORFs (3281) had homologues in common with both *S. cerevisiae* and *C. elegans* (Figure 3.4).

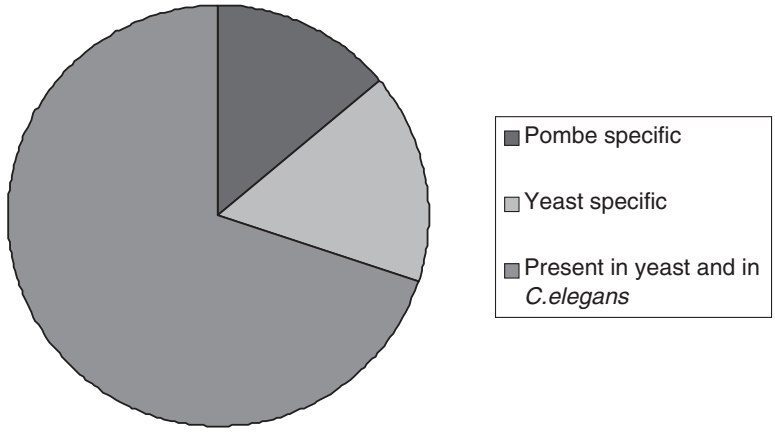

Figure 3.4 Using comparative genomics to assign biological significance to ORFs. Blast searches of all the *S. pombe* ORFs revealed that 681 ORFs (14 per cent) were uniquely present in *S. pombe*, 769 (16 per cent) were homologous to *S. cerevisiae* ORFs and approximately 3300 *S. pombe* ORFs (70 per cent) had homologues in common with both *S. cerevisiae* and *C. elegans*

With the availability of the *Neurospora* genome sequence it became possible to compare these single-celled yeasts with their multicellular cousins. Given that *Neurospora* produces at least 28 morphologically distinct cell types, and has a preponderance of 11 000 overwhelmingly non-paralogous genes, it is perhaps not surprising that a comparative genomic analysis reveals that a large proportion of *Neurospora* genes do not have homologues in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Analysis of other genome databases reveals that in relation to yeasts this particular filamentous fungus possesses an expanded group of sugar transporters, transcription factors and environmental sensing pathways and a diversified metabolic machinery: e.g. *Neurospora* possess 38 proteins with a cytochrome P450 domain whereas *S. cerevisiae* and *S. pombe* possess fewer than five such proteins.

As more fungal genomes become available it will become possible to identify subgroups of genes that are associated with specific types of fungal biology. Already, relatively crude comparisons between very distantly related fungi reveal much about the subset of fungal-specific genes, and genes that are yeast or multicellular specific. Ever more accurate predictions become possible as more genomes and indeed more closely related genomes become available. However, the power of comparative fungal genomics does not stop with revealing the biology of this important and ubiquitous group of organisms. Over 200 predicted *Neurospora* proteins exhibit significant similarity to human gene products associated with disease states in humans. Many of these have counterparts in *S. cerevisiae* and *S. pombe*, including 23 that are cancer-related proteins. Such findings lend support to the notion that fungi could be good model organisms for studying the associated human disease pathways.

Annotated genomes reveal much about the topology, evolutionary history and biological potential of cells, but not much about how this develops into an integrated biological entity. This requires a global view of how 2D DNA information manifests in 4D time and space. Experimental approaches to characterize global changes in cellular molecules, as they obey the central dogma (DNA makes RNA makes protein) have therefore been devised.

3.3 Transcriptomics and proteomics

3.3.1 Analysing encoded information in time and space

In the pre-genome era biologists used northern blot analysis of mRNA transcripts to explore the expression of the information stored in the DNA in the cell. Likewise they relied on pattern changes in 1D and 2D PAGE to monitor changes in cellular protein profiles. Researchers were limited at best to analysing time-lapsed gene expression profiles from a very limited number of genes, and piecemeal revelations as to precise 3D cellular context in which the encoded proteins carried out their functions. In the post-genomics world biologists exploit the availability of genome sequences to examine simultaneous

changes in gene expression profiles for every gene in the genome using array technology. Moreover, high-throughput protein analysis provides detailed information on cellular location, and protein–protein interactions, for thousands of characterized and uncharacterized proteins. The post-genomic era is characterized therefore by massively parallel molecular analyses of information-rich molecules.

3.3.2 Transcriptomics

If genomics deals with the relationship between DNA sequence and its encoded function, transcriptomics looks at RNA sequences and their expressed levels. If the pre-genomic technique of northern blot analysis of mRNA acted as an index of expression for one or two genes, the post-genomic world has access to a global perspective on gene expression patterns; and as was the case with eukaryotic genome analysis, global fungal transcriptome analysis began with *S. cerevisiae*.

3.3.3 Dissecting the diauxic shift using a yeast microarray

In a classic paper deRisi *et al*. PCR-amplified each of the 6400 distinct ORFs described in the first edition of the yeast genome and printed these unique DNA molecules onto glass slides using a simple robotic printing device. The resulting DNA microarrays, in which each of the known DNA sequences was attached at a particular grid reference on a 18 mm square area of a glass slide, was effectively a multi-gene probe. This was then used simultaneously to explore the expression profile of mRNA as yeast cells underwent a diauxic shift from fermentative growth to aerobic respiration. mRNA samples, taken over a 21 hour period as the cellular metabolism changed from glucose fermentation to ethanol respiration, were reverse-transcribed into cDNA molecules labelled with a red fluorescent dye. Green fluorescent cDNA was also prepared from the mRNA at the first time point to serve as reference. The expression level of each gene was measured by hybridizing fluorescently labelled cDNA to the probe, visualizing the fluorescence pattern using a confocal microscope and using a computer to analyse the image the relative intensity of the spots. At the initial time point, the green and red signals were equal, and all spots therefore appeared yellow. At later time points, red indicated that gene expression had increased relative to the reference, whereas green indicated that gene expression was lower in that sample than in the time zero control (Plate 2).

The analysis revealed that a staggering 28 per cent of all yeast genes underwent a significant alteration in gene expression level as a result of a diauxic shift: 710 genes were induced and 1030 genes repressed by a factor of two or more. Moreover, cluster analysis identified groups of genes whose pattern of expression changed in association with one another. Such co-ordinated gene regulation points towards a common promoter element – a co-ordinated regula-

tion that helps to identify possible cellular roles for ORFs that encode proteins of unknown function. For example, the sequences upstream of the named genes in Plate 3 all contain stress response elements (STRE). When the promoter sequences of the 13 additional (not previously recognized as stress-inducible) genes that shared this expression profile were examined, nine were found to contain one or more recognizable STRE sites. This suggests that many of these 13 additional genes, which were unidentified heretofore, have a role that is linked to stress response.

The same DNA microarrays were also used successfully to identify genes whose expression was affected by deletion of the transcriptional co-repressor *TUP1* or overexpression of the transcriptional activator *YAP1*, thereby demonstrating the feasibility and utility of this approach to the dissection and characterization of regulatory pathways and networks on a genome-wide basis.

3.3.4 The vocabulary of transcriptomics

Encouraged by the successful demonstration of this approach to global gene expression studies, other yeast studies rapidly followed. Then as genomes became available other fungi were explored in the same way. Fungal researchers found themselves embracing the new vocabulary of post-genomic analyses as they set about exploiting this approach in their organism of choice. Copy DNA (cDNA) libraries, expressed sequence tags (ESTs), serial analysis of gene expression (SAGE), macro- and microarrays, BLAST searches and sequence alignments (Table 3.4) all entered the fungal literature within 5 years of deRisi's paper. Although *S. cerevisiae* led the way in the microarray-based analysis of fungal transcriptomes because it was the first available fungal genome, and also because its genes lack introns (therefore allowing the ORFs to be PCR amplified directly from genomic DNA), a number of other global approaches to mRNA analyses are also available. These include methods based on cDNA, SAGE and EST analysis. Microarrays carrying many or all of the genes for a wide variety of fungi are now available, and the questions that they are being used to address are of both the basic and applied varieties.

3.3.5 From clocks to candidosis – the 'when' of inherited information

DeRisi's seminal paper revealed the power of transcriptomics in dissecting out complex differential gene expression *when* cells are undergoing diauxic growth and *when* regulatory gene expression levels are altered. In short, it reveals the information stored in the genome as a dynamic process. This technology allows scientists simultaneously to analyse the expression levels of any number of individual genes as they change in time, thereby both identifying co-ordinated gene

Table 3.4 Post-genomic vocabulary

BLAST (Basic Local Alignment Sequence Tool)	An algorithm that searches a sequence database for sequences that are similar to the query sequence. There are several variations for searching nucleotide or protein databases using nucleotide or protein query sequences
Complementary DNA (cDNA)	A DNA molecule synthesized by reverse transcriptase using an mRNA molecule as template. Hence, cDNA lacks the introns found in genomic DNA
Complementary DNA library	A collection of clones each containing a cDNA derived from the RNAs isolated from a specific tissue or cell and inserted into a suitable cloning vector
Expressed sequence tags	Short cDNA sequences that are derived from sequencing of all the mRNAs present in a cell. ESTs represent the expression profile of the cell at the time point of RNA isolation
Microarray	An ordered grid of DNA sequences fixed at known positions on a solid substrate, e.g. glass slide
Open reading frame	The sequence of codons, in DNA or RNA, that extends from a translation start codon to a stop codon
SAGE (serial analysis of gene expression)	Extremely short ESTs that are linked together as DNA chimeras consisting of 15 base pair sequences from 40 different mRNAs. The sequence analysis of thousands of these 40×15 bp chimeras permits a quantitative estimation of the mRNAs in the original sample
Sequence alignment	A linear comparison of nucleotide or protein (amino-acid) sequences. Alignments are the basis of sequence analysis methods and are used to identify conserved regions (motifs).

expression, and providing a window onto what happens *when* environmental changes and gene perturbations impact on the flow of information through the cell.

Knowing the 'when' of a gene's expression often provides a strong clue as to its biological role. Serial analysis of gene expression (SAGE) and microarray technologies have already been used: to identify the set of fungal genes most highly expressed during cerebrospinal fluid infection by *Cryptococcus neoformans*; to identify the pattern of differential gene expression that explains why glucose metabolism by *Trichoderma reesei* is so different to that of *S. cerevisiae*;

and to identify 18 genes of unknown function implicated in the production of carcinogenic aflatoxins by *Aspergillus parasiticus*.

Conversely, the pattern of genes expressed in a cell '*when*' it has been subjected to various treatments can identify critical points of great biological significance. This approach has been used to identify potential antimycotic targets to combat the increasing threat posed by candida infections. Microarray analysis has been used to identify two transcription factors *EFG1* and *CPH1* induced when *C. albicans* is shifted to 37 °C in the presence of serum, to establish that the final transcriptional profile found in some clinical isolates resistant to fluconazole could also be reproduced by allowing resistance to evolve in the laboratory and to demonstrate that the antimycotic itraconazole affected the expression of 296 ORFs in this fungus.

In some cases transcriptomics has been used to accelerate research projects that could be solved without this technology, but in other cases it allows fungal biologists to explore biological phenomena that are quite simply too complicated to approach in any other way. Moreover, this applies to fungal biology at both basic and applied levels. In an example of basic research *Schizosaccharomyces pombe* workers used expression data for each *pombe* gene to reveal that many conserved genes are expressed at high levels, whereas a disproportionate percentage of the poorly expressed genes are organism specific. This analysis was only possible because the expression data were available and database searches were possible of all ORFs in *S. pombe*, *S. cerevisiae* and the nematode worm, *C. elegans*. In another example of basic research that was impossible using pre-genomic methods, microarrayed cDNA sequences of 1000 different genes revealed that circadian clock control in *Neurospora crassa* covers a range of cellular functions rather than preferentially belonging to specific pathways.

An impressive example of applied research, on the other hand, is a microarray produced by the Agilent company. The first of its kind, this array carries 7137 rice ESTs, and all 13 666 predicted ORFs from the major rice fungal pathogen *Magnaporthe grisea*. It therefore uniquely allows simultaneous analysis of gene expression profiles in both the host and pathogen.

And then sometimes, unexpectedly, what was basic research to begin with turns out to have applied aspects: microarray analysis in *C. albicans* of the genes induced by α-factor in mating competent type a cells revealed 62 genes. Interesting in their own right as an insight into the cryptic mating pathway of this fungus, the realization that seven of these genes encoded cell surface or secreted proteins that had previously been shown to be required for full virulence of *C. albicans* unexpectedly revealed new potential targets for the development of antimycotis aimed at the factor(s) regulating the mating pathway.

In short, parallel analyses of global expression patterns, which strives to project 2D genomic DNA sequence information into a time-related 3D organization, is revolutionizing the way fungal biologists do science.

3.4 Proteomics

2D and 3D analyses of biological information are extremely powerful. However, it is the 4D manifestation of that information in time and space that biologists seek to define and understand. Small wonder that proteomics, which directly addresses that extra dimension, faces challenges that are orders of magnitude beyond those posed by the global analysis of the 2D and 3D information present in DNA and mRNA respectively. One has only to look at Table 3.5 to realize that, unlike genomics and transcriptomics, proteomics is in fact an entire suite of distinct but intricately interrelated technologies. Even though gel electrophoresis provided a platform for the global analysis of cellular proteins as early as 1978, it revealed nothing of the spatial arrangement of the proteins in the cell. Furthermore it revealed little about post-translational modifications, only visualized moderately abundant proteins, and told the researcher nothing of the protein's structure and function. Developments such as micro-sequencing of the gel-separated proteins, and sensitive glyco-specific stains, addressed some of these limitations in the pre-genomic era, but global analysis of proteins in the post-genomic era seeks to build on genomic and transcriptomic projects to provide databases of information that address all of the parameters listed in Table 3.5 and more.

3.4.1 Protein sequence and abundance

At its most basic level proteomics seeks to identify, at a given moment in time, which ORFs in a genome are expressed as proteins in a cell, and what level of each one is present. Unlike RNA and DNA sequences that can be enzymatically amplified, large-scale parallel analysis of proteins must deal with unavoidable

Table 3.5 Proteomics is much more challenging than transcriptomics or genomics

DNA (information)	RNA (information in time)	Protein (information in time and space)
Nucleotide sequence	Nucleotide sequence Level in cell Modifications (splicing)	Amino-acid sequence Level in cell Modifications (glycosylation, phosphate groups etc.) 3D structure Function Location Interactions

problems of limited sample material and abundance variation over six orders of magnitude. Nevertheless, a combination of tryptic digestion of protein mixtures, coupled with chromatographic separation of the resulting peptides, allows increasingly sophisticated mass spectrometry to identify the presence and abundance of ever smaller amounts of protein from increasingly complex protein mixtures. Peptide fingerprints (the spectrum of ionized molecules generated by the peptides in an MS system) can then be used to identify the same pattern already entered into the databases from MS analysis of known proteins. Alternatively, a further round of mass spectrometry can be performed on each peptide to identify its constituent amino acids and these sequences can be compared to entries in protein and DNA databases using the appropriate database searching tools. In a lower-throughput system, protein mixtures can first be separated by 2D gel electrophoresis, protein spots excised, digested with trypsin and subjected to MS analysis in the same way.

This type of proteomic analysis, which essentially generates the same type of information on the protein level as transcript analysis does at the RNA level, has already been used to probe fungal biology. 2D electrophoresis followed by MS analysis was used to assess the effect of concanamycin A, an antibiotic produced by *Streptomyces*, on protein levels in the filamentous fungus *Aspergillus nidulans*. Twenty spots were identified and five excised for tryptic digestion followed by estimation of their complete amino-acid sequence by mass spectroscopy. The functions of four of those proteins were identified using the protein sequence to search ORF databases; a fifth was identified as being homologous to a protein in *A. niger*, but of unknown function. In a more extensive analysis approximately 100 extracellular proteins secreted by *A. flavus* when provided with the flavonoid rutin as the only source of carbon were identified by 2D electrophoresis. Trypsin digestion and MS analysis allowed 22 of these to be identified by searching peptide fingerprint databases, but over 90 protein spots remained unidentified, indicating that these proteins are either novel proteins or proteins whose peptide fingerprints are not yet available.

In common with other organisms, a recurring theme in protein sequence and abundance analyses is that proteomic findings differ with respect to transcriptomic findings. In some cases mRNAs are found but no corresponding protein, indicating either that the protein was in very low abundance or unstable during extraction, or that those transcripts were the subjects of post-transcriptional regulation. On other occasions proteins are present but no corresponding RNA is found – indicating a less than representative RNA sample, or a protein with an unusually long half-life. However, despite these differences large-scale parallel protein analyses are becoming a central theme in the fungal community. Indeed, so rapid has progress been that in the case of *S. cerevisiae* proteomics is now attempting to address the much more formidable question of how the proteins present in the cell interact with one another to form complexes, and how complexes are organized on a cellular scale.

3.4.2 From locations to interactomes

Once the presence and abundance of a protein has been determined for a given situation, the next parameter in the 4D arrangement of biological information is the cellular location of the protein in question. Thereafter, a more complete description requires information on (1) the proteins that it interacts with, (2) the complexes it is involved with, and (3) how these complexes interact within the cell. The yeast *S. cerevisiae* is currently in the forefront of turning this dream into reality. This is because it is an extensively characterized model organism with a well developed gene expression system, and its extremely accurate homologous recombination system allows novel gene constructs to be precisely targeted into the correct chromosomal locus, thereby ensuring that the gene is subjected to appropriate native gene regulation. Moreover, the vast majority of its ORFs are known and many have been characterized.

Using these cellular attributes Won-Ki Huh *et al*. used PCR to amplify hybrid DNA molecules consisting of the coding sequence for GFP fused in frame with the 3′ end of the coding sequence for each one of 4100 yeast ORFs (Figure 3.5).

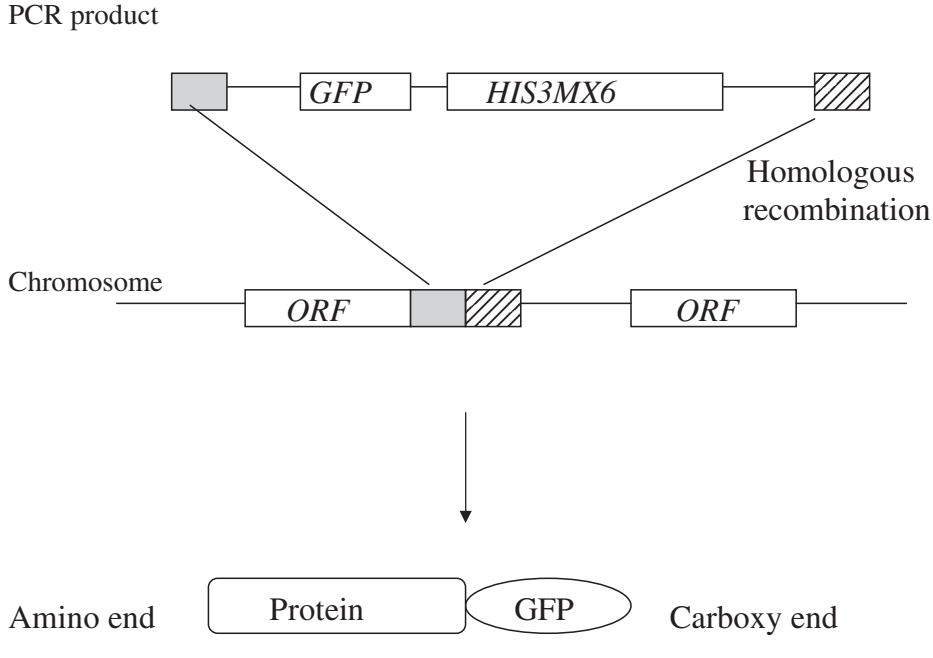

Figure 3.5 Creating a fluorescently labelled protein. PCR was used to create a hybrid DNA consisting of a selectable marker (*His 3*) and the coding sequence for GFP fused in frame with the two adjacent sequences of DNA from the 3′ end of the coding sequence of the target ORF. Homologous recombination targets the hybrid molecule back into the correct chromosomal locus thereby creating a GFP tagged gene sequence

They then used homologous recombination to target each hybrid molecule back into the correct chromosomal locus, thereby creating 4100 yeast strains, each one carrying a different GFP-tagged gene sequence. Then using fluorescence microscopy to find out where they were in the cell they managed to classify these proteins into 22 distinct sub-cellular locations and in doing so provided localization information for 70 per cent of previously unlocalized proteins – a number that constitutes about 30 per cent of the yeast proteome.

Hybrid DNA molecules encoding fusion proteins were also the basis of the first systematic search for interacting proteins in a yeast cell. Using a slight variation of the two-hybrid system, described in detail elsewhere in this book (see chapter 6 (section 6.6.2), they mated 192 cells expressing different 'prey' proteins with each of 6000 strains expressing different 'bait' proteins and used reporter gene expression in the resulting diploid cells as an index of interaction. Although all two-hybrid studies identify false positive interactions and fail to identify weakly interacting protein ones, they do provide strong clues as to possible protein interactions and functions. In this study two proteins of unknown function were found to be intimately linked to arginine metabolism, a heretofore-unknown cell cycle control circuit was discovered, and small networks of interactions traced a series of protein interactions joining a protein involved in the formation of double-stranded DNA breaks and one involved in the formation of the synaptonemal complex during meiosis. In a subsequent paper using these and related data from other studies Schwikowski *et al.* compiled a list of about 2700 protein interactions in *S. cerevisiae* and found that 1548 yeast proteins could be depicted in a single large network (Figure 3.6). Moreover, within the network it was found that proteins that could be allocated to specific cellular functions (e.g. DNA synthesis, amino-acid metabolism, structural proteins etc.) showed a high level of interactions and therefore clustered together into functional groups. This allowed them to ascribe potential functions to almost 40 yeast proteins previously of unknown function. Their global interactome revealed the 'great interconnectedness' of the yeast proteome. It found that many members of protein clusters associated with central roles (e.g. cell cycle) had interactions with proteins from many other cluster classes (e.g. DNA synthesis, transcription, polarity, etc.), thereby connecting biological functions into larger cellular processes. On the other hand, not many members of functional clusters for more peripheral cellular functions (e.g. RNA turnover) interacted with proteins from other clusters. This meta-analysis allowed a meta-network consisting of 32 functions (and their 70 or so associated interlinking connections) to be described in place of 1548 individual proteins and their 2700 links. The graphical representations of protein-interaction maps provide a rough outline of the complexity of protein associations.

Despite its power, this type of analysis lacks biological context beyond the possibility that the two proteins in question can interact given the artificial circumstances of being constitutively expressed at high levels in the same cellular compartment at the same time. However, a powerful complementary yeast study

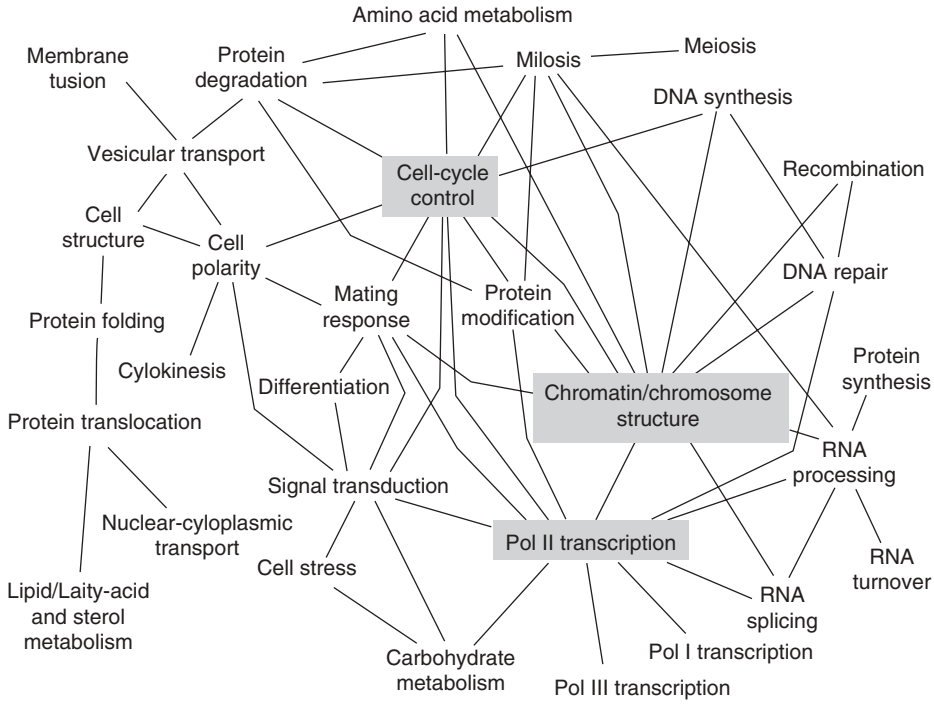

Figure 3.6 A diagram of the meta-network consisting of 32 functions (and their 70 or so associated interlinking connections) in place of the 1548 individual proteins and their 2700 links that constitute the yeast interactome

rectified all that: Gavin *et al.* exploited homologous recombination to integrate hundreds of ORFs fused to a TAG into their normal regulatory sequences. The transformed cells were then allowed to express the modified proteins before appropriate chromatography was used to isolate the tagged protein and with it, in many cases, complexes of other proteins with which it had been interacting. The proteins extracted with the tagged bait were identified using standard mass-spectrometry methods. This approach identified 1440 distinct proteins within 232 multi-protein complexes in yeast. This study provided new information on 231 previously uncharacterized yeast proteins, and on a further 113 proteins to which the authors ascribe a previously unknown cellular role.

Powerful though these types of study are, it is estimated that over 70 per cent of all protein–protein interactions in yeast still remain undetected. Undoubtedly future studies will reveal many of these, but blindly executed global analyses are unlikely to find *all* of the subtle interactions that pertain in this organism.

Therefore, identifying the 4D arrangement of all the intricate cellular protein interactions, complete with biochemical kinetics, is still a long way off. Nevertheless, a complementary strategy uses information that already exists in databases to develop theoretical models of the interactions between collections of elements responsible for carrying out well defined biological tasks. Such models can provide a rational framework in which to design a focused range of experiments – the data from which can in turn be used further to refine the model. Such attempts to re-integrate molecular details to reveal the secrets of the dynamic processes they mediate within the cell forms an experimental and theoretical approach referred to as *systems biology*.

3.5 Systems biology

3.5.1 Establishing cause and effect in time and space

The post-genomics era has been dominated to date by gigantic data-accumulating exercises – data that allows us to analyse encoded information in time and space. Now the challenge is to reintegrate this data to provide meaningful insight into biological phenomena.

3.5.2 Case study: the application of systems biology to modelling galactose metabolism – a basic fungal metabolic pathway

The process of galactose utilization has been extensively studied for many decades at both the genetic and biochemical levels in the yeast *S. cerevisiae*. Induced by galactose in the absence of glucose, the nine genes, the gene products (one transporter protein, four enzymes and four transcription factors), metabolic substrates and key gene regulatory networks have all been worked out during 30 years of pre-genomic experimentation (see the solid lines in Figure 3.7).

In *step 1* of this systems approach all of this information was used to define an initial model of galactose utilization. In *step 2* each pathway component was systematically perturbed, yielding 20 separate cellular conditions. These were wild-type cells and deletion strains for each of the nine genes grown in the presence and absence of galactose. The global cellular response to *each perturbation* was detected and quantified using array technology. Also in the case of the wild-type cells large-scale protein expression analysis was performed. In *step 3* all of this new information was integrated with the initially defined model and also with the information currently available on the global network of protein–protein and protein–DNA interactions in yeast.

Amazingly, this microarray analysis revealed that mRNA synthesis was significantly altered in the case of 997 yeast genes (i.e. approximately 20 per cent

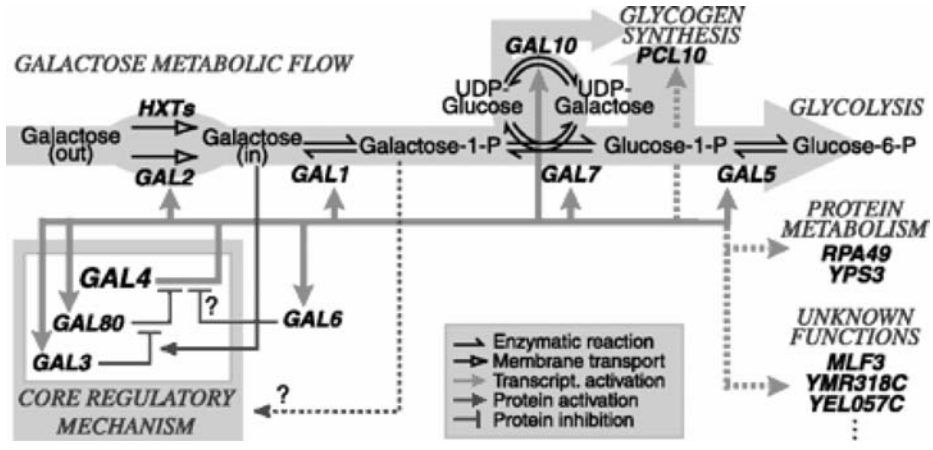

Figure 3.7 System analysis reveals new levels of regulation in a basic fungal metabolic pathway. Dotted interactions denote model refinements to galactose metabolism in yeast following a post-genomic global analysis of the yeast genome sequence, microarray-based mRNA analysis, databases of protein–protein interactions, and rapid MS analysis of proteins

of all ORFs) in one or more of these perturbations, and this despite the fact that only nine gene products are needed for galactose metabolism. It would therefore appear that the 'great interconnectedness' of the yeast protein interaction network is extensively perturbed by a handful of perturbations to the galactose metabolic system.

In *step 4* the researchers formulated new hypotheses to explain the observations not predicted by the model, and then designed and executed additional perturbation experiments to test the new 'improved' model. For example, they detected an unexpected reduction in the expression levels in GAL enzyme genes (suggesting a regulatory effect) in cells carrying a deleted gal7 gene (encodes the enzyme responsible for converting galactose-1-P to glucose-1-P). This suggests the presence of an additional heretofore unknown regulatory circuit, which down-regulates GAL gene expression when galactose-1-P accumulates in the cell. This new 'improved' model was tested by additionally deleting gal 1 (which encodes the enzyme that converts galactose to galactose-1-P). As predicted this essentially reversed the effect.

This seminal systems biology paper was only made possible because the yeast genome had been sequenced, microarray analysis of yeast mRNA had been developed, databases of protein–protein interactions were available, rapid MS analysis of protein presence and abundance was possible, and computing facilities to process all of the information were available. In short, systems biology is only possible in a post-genomic world.

3.6 Conclusions

From one gene–one enzyme in *Neurospora* to the cell cycle in yeast, fungal experiments have been at the forefront of altering the way biologists view their world. Yet the sum total of all the great discoveries in fungi from Beadle and Tatum to Hartwell and Nurse are set to pale into insignificance in the face of the potential offered by fungal systems biology in this, the *post-genomic era*. Led by *S. cerevisiae* there is a paradigm shift towards *massively parallel comparative biology* in all types of organismal research. Even within the context of this book, such an approach offers rapid progress in every area of this field including exploring native and transgenic fungal biology, identifying genomic differences for strain improvement, understanding the molecular consequences of fungal infection and identifying targets for the development of new antimycotics. The opportunities offered by post-genomic research are quite simply limitless.

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3.8 Revision questions

Q 3.1 Why is yeast a cornerstone of post-genomic research?

Q 3.2 What is the fundamental difference between pre- and post-genomic research?

Q 3.3 What is systems biology?

Q 3.4 What is an open reading frame (ORF)?

Q 3.5 How many 'new' ORFs were discovered when the yeast genome was analysed, how many of the 6000 ORFs were initially annotated with functions, and how many have had functions determined since then?

Q 3.6 What does ORF redundancy reveal about the dynamics of genetic information in *S. cerevisiae*, *S. pombe* and *N. crassa*?

Q 3.7 Provide a brief description of the three basic approaches to identifying the functions of newly discovered ORFs.

Q 3.8 Explain how deRisi *et al.* fabricated and used the first yeast microarray.

Q 3.9 Explain the meaning of BLAST searches, complementary DNA (cDNA) libraries, expressed sequence tags (ESTs), serial analysis of gene expression (SAGE) and sequence alignments.

Q 3.10 Outline the challenges faced by proteomics in defining 4D biology.

Q 3.11 How are fusion proteins made and used in yeast proteomics?

Q 3.12 Explain the 'systems biology' approach to understanding biological phenomena in yeast.

4

Fungal Fermentation Systems and Products

Kevin Kavanagh

4.1 Introduction

Fungi represent one of humanity's oldest domesticated organisms and are responsible for the production of some of our most enjoyable (e.g. alcohol), nutritious (e.g. bread) and medically useful (e.g. penicillin) products. Although not the subject of the material presented in this chapter, it should also be emphasized that fungi are extremely important decomposers of organic material such as leaf litter. Many fungi engaged in decomposition are also the producers of enzymes and antibiotics of commercial importance.

The use of yeast to make bread and alcohol has been recorded for thousands of years. The Babylonians (circa 6000 BC) and Egyptians (circa 5000 BC) have left written accounts of their production of beers, wines and bread – all of which warranted the use of yeast. Indeed, the ancient Israelites and Egyptians recognized the difference between leavened (used yeast to 'raise' the dough) and unleavened bread from as early as 1200 BC. Yeast cells were first observed microscopically by Van Leeuwenhoek in 1680 and in 1838 the yeast involved in brewing was called *Saccharomyces cerevisiae*. Although the biochemical role of yeast in the fermentation process was not elucidated until 1863 by Louis Pasteur, the products of the process have been enjoyed for millennia!

Apart from their role in providing humans with food and alcoholic beverages for thousands of years, fungi have had a significant impact on the course of human history. The Roman Empire was one of the greatest empires the world has ever seen and the reasons for its demise has been a source of much debate.

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Recent evidence suggests that one factor that may have accelerated the decline of Rome was the reduced yield from cereal crops due to a series of warm, humid summers facilitating the growth of rusts and smuts (see Chapter 9). The reduced yield led to higher prices for bread with associated food riots. A similar scenario may have contributed to the French Revolution in 1789. The devastating famine of 1845–1848 in Ireland, which killed a million people and forced a million to emigrate, was due to potato blight caused by the fungus *Phythopthora infestans*. Fungi have also had beneficial effects on human history. The discovery of penicillin production by Alexander Fleming allowed the treatment of wound infections and subsequent improvement in patient survival. Prior to the D-Day landings in June 1944 the Allies developed stocks of penicillin to treat wounded soldiers – without this antibiotic the deaths from the European invasion would have been far greater and the liberation of Europe might not have been attempted.

4.2 Fungal fermentation systems

Fungi are employed to produce a wide range of foods (e.g. bread, mycoprotein), alcoholic beverages (wine, beer), recombinant proteins, vitamins and antibiotics (Table 4.1). To achieve this productivity, a variety of fermentation systems are employed with fungi – the choice of which will depend upon the nature of the fungus (filamentous or yeast), the type of product that is required and the scale of the production (Table 4.2). Yeast cells have a typical mean generation time (time for the population to double) of 1.15–2 hours while filamentous fungi (moulds) divide every 2–7 hours approximately; consequently, fermentation systems that are used with fungi may be of limited value for animal and plant cells, which have much longer doubling times. In addition, due to the difference

Table 4.1 Industrial applications of fungi

Product	Example
Biomass	Production of baker's yeast Brewer's yeast tablets Single-cell protein (mycoprotein)
Cell components	Proteins (native or recombinant)
Products	Antibiotics (penicillin) Vitamins (B_{12})
Catabolite products	Ethanol
Bioconversion	Breakdown of range of carbohydrates

Table 4.2 Fungal fermentation systems and products

Fermentation type	Example	Product
1. Solid	Cultivation of *Agaricus bisporus*	Mushrooms
2. Batch	Brewing, wine making	Beer, wine
3. Fed batch	Cultivation of *Saccharomyces cerevisiae*	Baker's yeast
4. Cell recycle batch	Wine making (Italy)	Summer wines
5. Continuous	Cultivation of *Penicillium graminerium*	Mycoprotein

in growth morphology between yeast and filamentous fungi some fermentation systems are suitable for use with the former cell type but not the latter. While a number of basic fermentation systems exist, modifications to these are often implemented to 'fine tune' a system to the production of a specific fungal product.

4.2.1 Solid fermentation

Solid fermentation occurs where the fungus grows on a solid substrate (e.g. grain) in the absence of free water. In this case the fungus must be able to tolerate low water activity. This type of fermentation system has been used for the production of edible mushrooms (*Agaricus bisporus*) and for the growth of filamentous fungi for the isolation of antibiotics and enzymes. Solid fermentation is also employed to produce soy sauce from soy beans using *Rhizopus* sp. and cheese may be produced from milk curd using *Penicillium* sp. The production of horticultural compost is also an example of a solid fermentation. A combination of yeast (*Candida lipolytica*) and filamentous fungus (*Chaetomium celluloyticum*) is used to degrade straw to produce fungal biomass. Solid fermentations have a number of applications but can be difficult to control, and it may be expensive to sterilize the raw material.

4.2.2 Batch fermentation

The most commonly employed system for the commercial cultivation of fungi is batch fermentation. This system has been used to produce alcoholic beverages and also for the production of industrial solvents such as ethanol and acetone. In batch fermentation the medium is inoculated at a low density with fungal cells. After a lag phase of minutes or hours, during which there is intense biochemical activity within the cells, the exponential phase of growth com-

mences, which is accompanied by maximal utilization of nutrients. As the nutrient level declines the rate of population growth slows, until, in the stationary phase, there is no net increase in cell number and the population stabilizes. Prolonged incubation can result in a decrease in cell viability as cells die and the population declines. Ethanol production by brewing yeast strains is maximal as the culture enters the stationary phase; consequently, brewers monitor the appearance of ethanol and halt the fermentation when levels have reached a peak. Continuation of the fermentation will ultimately result in depletion of the ethanol as this is consumed by the yeast in the absence of the original carbohydrates. The culture will consequently display a bi-phasic growth curve.

Fed batch is very similar to batch fermentation except that nutrients are added periodically during the fermentation process. This type of system is optimal for maximizing biomass and is employed for the production of baker's yeast. Another variation of batch fermentation is called the Melle-Boinot process or cell recycle batch fermentation (CRBF). In this system the cell mass at the end of a fermentation is used to inoculate the next fermentation, thus ensuring a high cell density throughout the process. By maintaining a high cell density more energy is available for ethanol production and the yeast cells become adapted to produce and tolerate ethanol at a higher level. CRBF typically proceed for weeks or, in some cases, months, with each new cycle being inoculated with the cells from the previous cycle. The Melle-Boinot process is yeast strain and substrate specific but is used for the production of summer wines in some regions of Italy and is an efficient system for the production of ethanol from xylose (a five-carbon sugar) by the yeast *Pachysolen tannophilus* (Figure 4.1). In this latter case the elevated ethanol production is accompanied by an enhanced ethanol tolerance. Interestingly, CRBF has no significant effect on ethanol production from glucose by *P. tannophilus*.

4.2.3 Continuous cycle fermentation

The other main fermentation system used with fungi is continuous cycle. In this system cells are grown under steady state conditions and maintained at a particular stage of their growth cycle. Nutrients are added at a constant rate and biomass or spent medium is removed at the same rate in order to maintain a constant reaction volume. While continuous cycle fermentation has traditionally been difficult and expensive to establish for fungi, it is now routinely employed for producing mycoprotein and antibiotics.

4.2.4 Fungal cell immobilization

In addition to the different fermentation systems described above there is also the possibility of immobilizing fungi in order to optimize productivity. Cell

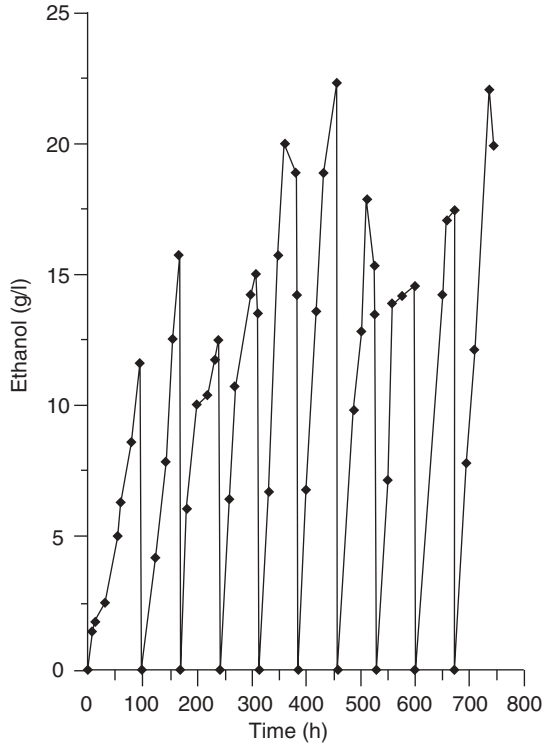

Figure 4.1 Cell recycle fermentation for the production of ethanol from xylose by the yeast *Pachysolen tannophilus* (at the end of each fermentation cycle cells are harvested and introduced into fresh fermentation medium)

immobilization ensures that a high cell density is maintained and that cells are not washed out of the bioreactor, it reduces the opportunity for contamination and removes growth inhibition due to the production of a toxic metabolite such as ethanol. The main disadvantage of immobilization is that fungal cell viability decreases over time and that the immobilization system may degrade with continual usage.

Four basic immobilization systems are used with fungi. The first system involves the containment of the fungal cells in a membrane such as dialysis tubing, microfilters or other porous material (Figure 4.2(a)). One problem encountered with this type of system is membrane clogging, which reduces the efficacy of the system.

Yeast cells may also be attached electrostatically or covalently to solid surfaces (Figure 4.2(b)). In one such process gluteraldehyde is employed to cross-link cells and attach them to a support, which can be immersed into growth medium or used as a substrate over which medium is passed.

Fungal cells have frequently been immobilized by entrapment in alginate beads (Figure 4.2(c)). In this case the cells are resuspended in a 2–4 per cent

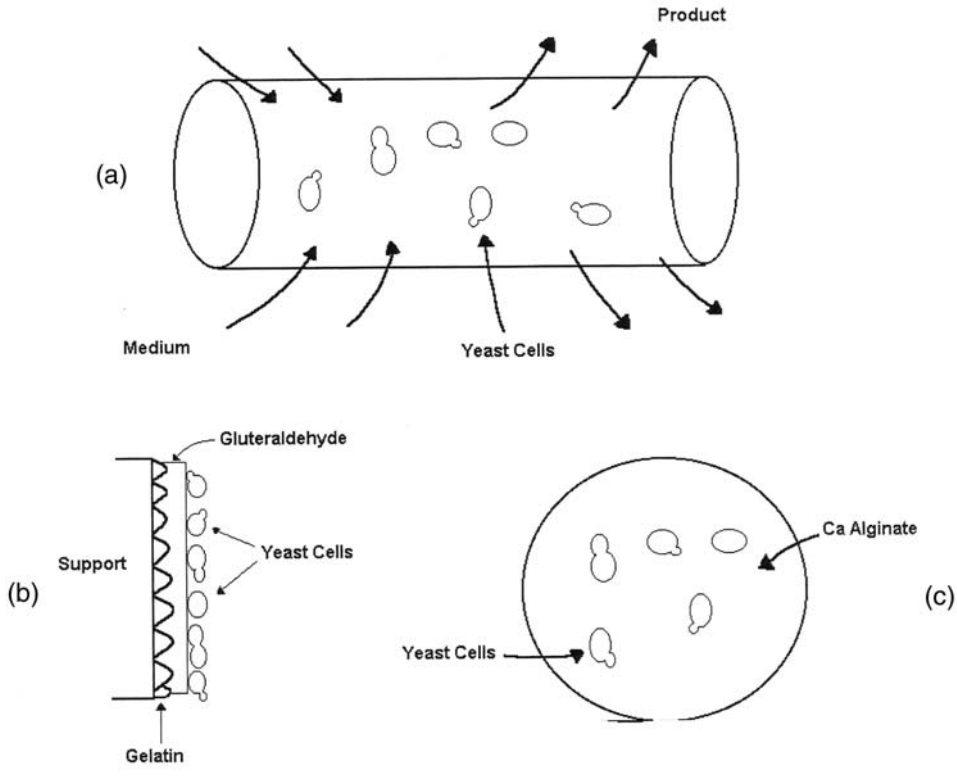

Figure 4.2 Fungal cell immobilization methods (a) containment of cells in membrane bioreactor (b) attachement to solid substrate (c) entrapment of cells in alginate bead

(w/v) sodium alginate solution, which is dropped into a calcium chloride solution, where insoluble calcium alginate is produced in the form of spherical beads of varying diameters. Such beads are biochemically inert, porous and stable, and entrap the fungal cells. Immobilization of yeast in alginate beads has led to an increase in ethanol productivity but has the disadvantage that the build-up of gas (especially carbon dioxide) within the bead may lead to disruption. Recently, other matrices such as agar, gelatine and polyacrylamide have been used for yeast entrapment.

The final form of immobilization involves inducing yeast cell flocculation or the formation of clumps. Flocculation is a natural process that normally occurs at the end of a fermentation. Inducing it at the end of a fermentation ensures a high cell density (potentially up to 10^{11} or 10^{12} cells/ml) and removes the need to centrifuge large volumes of culture medium to harvest cells.

Perhaps the most widely used system for immobilization is that which involves entrapping cells within membranes. This has a number of advantages including the ability to ensure a high cell density, to raise ethanol productivity and to maintain high dilution rates in continuous cycle systems, since cell run-off is not

a problem. Entrapped systems may present a diffusion barrier to the escape of metabolites through the surrounding matrix. In addition, there is the possibility that the metabolism of the yeast may change and affect the nature or quality of the desired product. Cell viability may be adversely affected in entrapped systems since any toxins produced by the cells cannot diffuse away as easily and hence exert a direct impact on cells within the matrix.

4.2.5 Downstream processing

Fungal fermentations can be employed to yield a range of products but in many cases these must be concentrated before use. Typically antibiotics and lipids are produced at a level of 10–30 g/l, single-cell protein at 30–50 g/l and ethanol at 70–120 g/l. Downstream processing (DSP) ensures that the product of interest is recovered in a reliable and continuous manner and that there is a large reduction in volume. The use of downstream processing will depend upon the nature of the product; for example, in the case of whisky, distillation is the main form of DSP. In the case of isolating proteins, the cell must be disrupted either by sonication, enzymatic degradation of the cell wall or compression in a French press. Nucleic acids from the lysed cell are degraded using a nuclease. The enzymes of interest can be collected by ammonium sulphate precipitation and purified by running through a variety of columns containing Sephadex, DEAE-Sephacel or hydroxy apaptite. Once the product has been recovered it must be prepared for marketing, possibly by mixing with other agents and by packaging.

4.2.6 Factors affecting productivity of fungal fermentation

Irrespective of the nature of the fermentation system chosen or whether cells are free or immobilized, a number of parameters affect fungal productivity. Specific process requirements will dictate the choice of a fermentation system. For example, ethanol production is well understood by batch fermentation but would be very poor if film fermentation were attempted. The nature of the fungal cell is critical, since yeast cells are amenable to growth in a bioreactor that uses spargers (paddles) to ensure nutrient mixing or oxygen transfer, whereas spargers would disrupt the mycelium of a filamentous fungus and adversely affect the fermentation. Agitation of fungal mycelium in bioreactors is usually achieved by bubbling air or gas (nitrogen) through the medium (Figure 4.3). Some fermentations require aerobic conditions while others must be performed under semi-aerobic or anaerobic conditions. Certain fermentations require a specific initial inoculum in order to grow at an optimal rate. The nature of the growth medium must also be optimized for efficient fermentation to occur

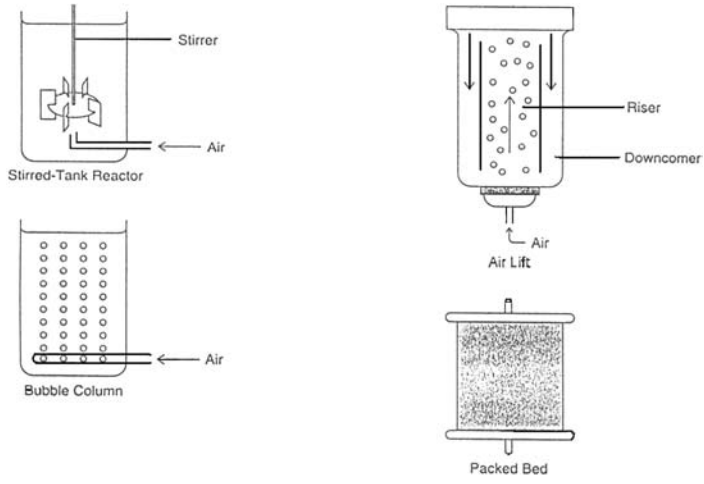

Figure 4.3 Means of aerating fungal cell cultures

– many fungi are subject to catabolite repression consequently too high a concentration of glucose can inhibit the process. The temperature of the fermentation affects productivity and a specific temperature can determine, for example, whether an ale or lager is produced during brewing.

Despite the wide range of fermentation systems available, the majority of commercially important fermentations are still either batch or fed batch. Part of the reason for this is that these systems are relatively easy to set up, the batch size can be varied to meet demand and historically most fermented beverages have been produced by batch fermentation. In addition, in the case of brewing the yeast cells are discarded at the end of the fermentation and a new inoculum is used, thus ensuring that strain specific characteristics associated with a particular beer are not lost during prolonged fermentation. This is very important in ensuring the consistency of beers.

4.3 Ethanol production

Yeast are capable of producing ethanol by the semi-anaerobic fermentation of sugars, as in the case of beer, wine or cider manufacture. In addition to the production of ethanol, yeast also produce a range of compounds known as organoleptics, such as isoamyl alcohol, ethyl acetate and butanol, which give flavour to the final product. During the fermentation process yeast convert glucose ($C_6H_{12}O_6$) initially into pyruvate through the process of glycolysis. Pyruvate is converted to acetaldehyde by the action of the enzyme pyruvate decarboxylase with the release of carbon dioxide. Acetaldehyde is subsequently

converted to ethanol ($2C_2H_5OH$) by the action of alcohol dehydrogenase. The overall reaction can be summarized by the following equation:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad \Delta G^\circ \text{ (fermentation)} = -58\,\text{kcal/mol}$$

4.3.1 Brewing

Wine and cider are produced from grapes and apples, respectively, where the fermentable carbohydrates are simple sugars such as glucose or fructose. In the case of beer production there is an added complication in that the carbon source in barley, starch, is non-fermentable by most yeast, which lack amylases and glucoamylases. In order to obtain a fermentable matrix the barley is first germinated in the process of malting. During malting the endogenous α, β and gluco-amylases convert the starch into groups of fermentable sugars, which the yeast can subsequently metabolize to ethanol (Figure 4.4). α-amylase acts internally on the β1,4 bonds in the starch molecule to form oligosaccharides of seven to 12 glucose units. β-amylase cleaves maltose units from the non-reducing ends produced on the oligosaccharide chains. The breakdown of starch to maltose and dextrins is known as saccharification. Once malting is complete, the barley seeds are dried and the nature of the drying affects the subsequent type of beer. A long cool drying process produces a 'pale' malt, which retains high enzymatic activity, whereas a short hot drying process produces a 'dark' malt, with low enzymatic activity. The dried seeds are crushed in the process of milling and the 'grist' is sprayed with hot water to extract the sugars and enzymes. At this point non-fermentable carbohydrates (e.g. starch) represent the majority of sugars in the 'wort' but over a relatively short time the action of amylases converts such carbohydrates to fermentable compounds. After 2–3 hours incubation fermentable sugars represent approximately 75 per cent of the sugars in a typical wort with non-fermentable carbohydrates constituting the remainder of the

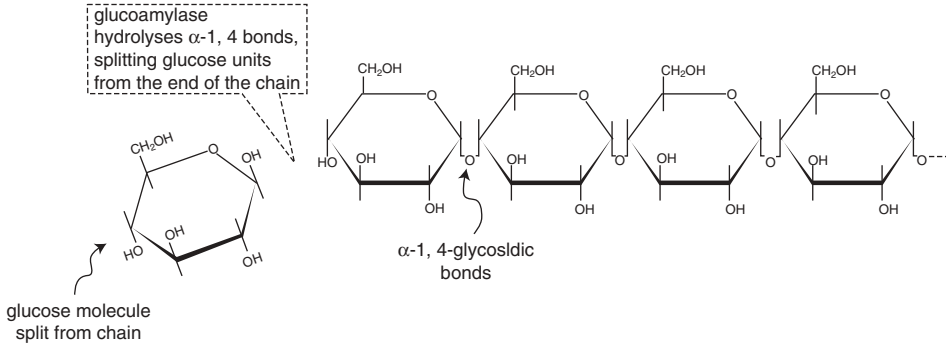

Figure 4.4 Breakdown of starch by amylases to yield a range of fermentable and non-fermentable sugars

sugars. A typical wort at this stage contains the following fermentable sugars: fructose (2.1 g/l), glucose (9.1 g/l), sucrose (2.3 g/l), maltose (52.4 g/l) and maltotriose (12.8 g/l). Non-fermentable carbohydrates account for 23.9 g/l. The wort is boiled to sterilize it and reduce the pH. Hops are also added to the wort during boiling to impart flavour and tannins to the beer, which can give stability to the 'head' on the beer.

The yeast inoculum is added to the cooled and filtered wort and the brewing process commences. Traditionally top fermenting yeast were used to make beer. These had an optimum temperature in the range 15–22 °C and rose to the top of the beer at the end of the process. Bottom fermenting yeast are now employed to produce the majority of beers; there have a lower optimum temperature range than the top fermenting yeast and settle to the bottom of the beer at the end of the fermentation in a process known as flocculation.

Brewing is a semi-anaerobic process, which can be controlled by regulating temperature and agitation. Controlling the size of the inoculum can also be used to regulate the 'speed' of the fermentation. The flavour of the beer is affected by the nature of the barley, whether a 'pale' or 'dark' malt is used, the type of hops and the ionic composition of the water in addition to the nature and amount of organoleptic compounds produced by the yeast during brewing. The ionic composition has a subtle influence on the nature of the beer. For example, Guinness produced in Dublin (Ireland) uses water with a low sodium content but a high carbonate content whereas the same beer produced in London (UK) uses water with a high sodium content and a lower carbonate level. These differences lead to slight variations in the taste of the final products. At the end of the brewing process beer is filtered to remove the yeast, pasteurized and packaged prior to dispatch. Some beers are left to mature prior to this final step (Figure 4.5).

Brewing can be used to give a range of beers depending upon the nature of the market to be supplied. Ales are typically produced using a fermentation temperature of 20–25 °C for 36 hours, after which the wort is cooled to 17 °C for 72 hours. In contrast, lager fermentations commence at 7–11 °C and can take up to 12–14 days to complete. Some beers, for example Budweiser, are produced using an extra source of amylases such as rice. Human saliva also contains amylase and this has been used to produce 'native beers' from sogrum or millet. In this case the grain is ground-up and human saliva is added. The amylases in the saliva break down the starch, releasing fermentable carbohydrates, which are fermented to ethanol by yeast. In recent years 'light beers' have become commercially available. In these, the non-fermentable carbohydrates (dextrins, starch) are converted to fermentable carbohydrates by the addition of exogenous amylases and glucoamylases, which are subsequently fermented to ethanol. In January 2004 the Neuzeller Kloster brewery in Germany announced the launch of an anti-ageing beer! This beer is supplemented with vitamins and minerals 'designed to slow the ageing process'. Time will tell whether this new product really does delay the ageing process!

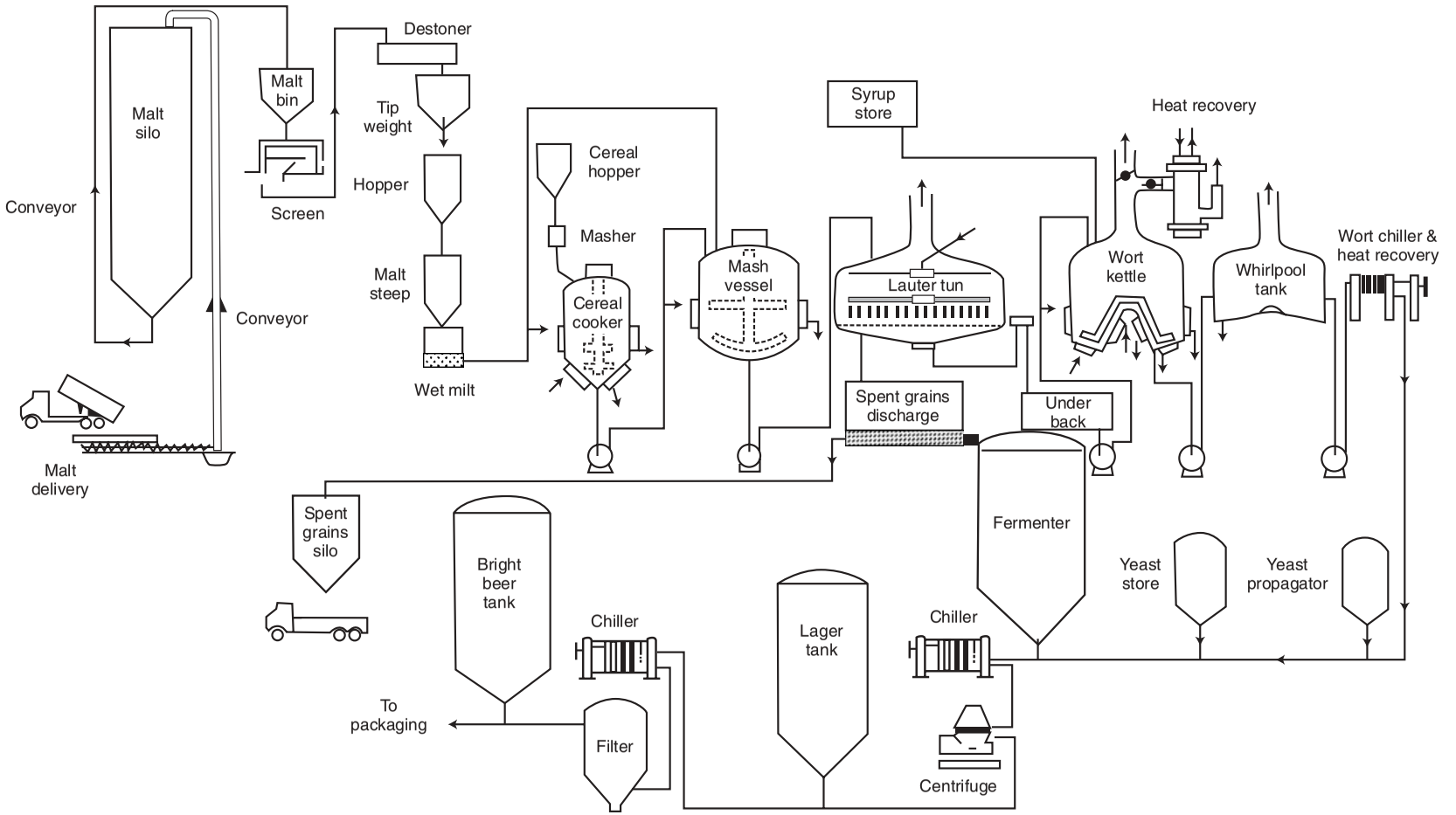

Figure 4.5 Flow chart of the stages in the production of beer

4.3.2 Recent advances in brewing technology

Although traditional methods of producing alcohol for human consumption are still employed, much attention in recent years has focused on improving the yeast to give a higher ethanol yield, a faster fermentation time or to reduce the production of unwanted off-flavours (Table 4.3). Genetic engineering has been employed to generate brewing yeast that convert α-acetolactate directly to acetoin without the involvement of diacetyl – an organoleptic compound that is responsible for unpleasant flavours in beer. The gene for α-acetolactate decarboxylase (α-ALDC) has been introduced into yeast from bacteria and this allows the conversion of α-acetolactate directly to acetoin without the formation of diacetyl (Figure 4.6). Pilot studies using genetically modified yeast show lower levels of diacetyl, but these strains are not yet used commercially. In addition, brewing yeast have been genetically engineered to incorporate the ability to produce amylase and glucanase, and to form clumps (flocculate) at a high frequency at the end of the brewing process. Variations in the brewing process have also led to enhanced ethanol production and faster fermentation times. Ethanol fermentations have been demonstrated under continuous culture conditions and immobilized cells have been employed as the yeast inoculum. In this case a higher cell density is attainable, there is less production of diacetyl and the fermentation is faster than when free yeast cells are used. While genetically engineered yeast have been shown to be superior to conventional yeast in many respects, their use for the production of commercial products has not yet occurred due in part to the reluctance of the consumer to purchase genetically engineered produce.

Table 4.3 Reasons for attempting genetic manipulation of brewing yeast

1. **Carbohydrate utilization**: utilize all remaining carbohydrate in beers.
2. **Increased product tolerance**: alter tolerance of yeast to ethanol and potentially increase yield.
3. **Temperature:** alter tolerance of yeast to high or low temperatures.
4. **Growth yield**: curtail growth of yeast culture but optimize ethanol production.
5. **Flocculence**: cultures of *S. cerevisiae* form clumps (flocs) at the end of the fermentation. May need to enhance this process.
6. **Organoleptics**: reduce or alter production of organoleptic compounds.
7. **By-products**: introduce the ability to produce vitamins, proteins into brewing strains.
8. **Novelty beers**: design yeast that will produce low-ethanol beers but retain full flavour of original product.

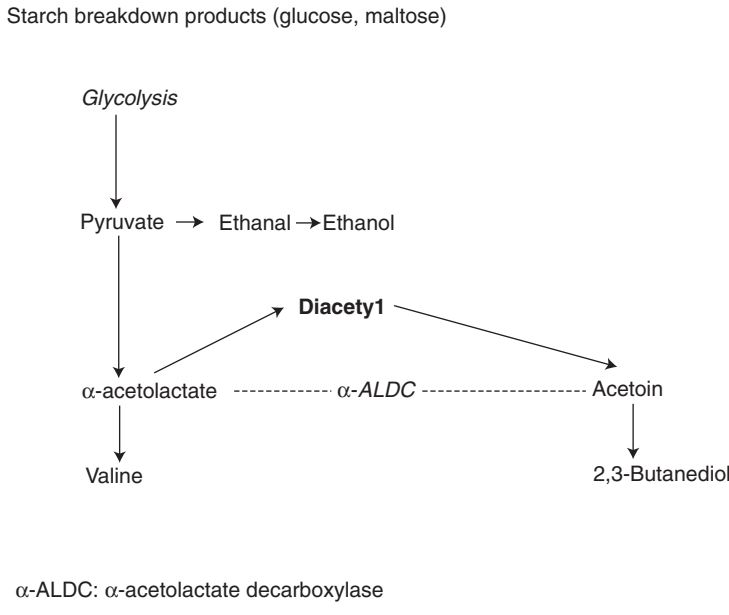

Figure 4.6 The introduction of α-acetolactate decarboxylase into brewing yeast prevents diacetyl formation during ethanol production

4.4 Commercial fungal products

4.4.1 Role of yeast in bread making

Yeast play a central role in the manufacture of bread. In this case the dough is infused with *Saccharomyces cerevisiae* and aerated. The dough is left to stand for a short period in a warm environment, during which time yeast cell respiration occurs and carbon dioxide is produced. Respiration can be summarized by the following equation:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad \Delta G°(\text{respiration}) = -686\,\text{kcal/mol}$$

The production of carbon dioxide gives bread its light, airy quality. While baking is essentially an aerobic process, some semi-anaerobic conditions develop in the dough and ethanol can be produced in small amounts. This, together with the carbon dioxide, is burnt off during the baking process and gives rise to the 'fruity' smell often associated with bakeries. In some parts of Japan bread dough is left to stand for a few days, during which the oxygen is used up in respiration and fermentation commences. The dough soon contains appreciable levels of ethanol and is subsequently eaten!

Table 4.4 Material and fungi used in single-cell protein (SCP) production

Material	Fungi
Cellulose	*Trichoderma viride*
Ethanol	*Candida utilis*
Banana peels	*Pichia* species
Beef fat	*Saccharomycopsis* species and *Candida utilis*
Kerosene	*Candida lipolytica* and *Candida utilis*
Sugars	Range of yeast species
Starch	*Saccharomycopsis fibuliger*

4.4.2 Single-cell protein – a novel food source

A range of fungi can be utilized to yield single-cell protein (SCP) from a variety of sources (Table 4.4). The filamentous fungus *Trichoderma viride* has been employed to obtain SCP from cellulolytic material, and yeast of the genus *Candida* are utilized to yield SCP when grown upon kerosene. The yeasts *Saccharomycopsis fibuliger* and *Candida tropicalis* are employed to produce SCP from starch in the 'Symba process'. In this process starchy effluents from food processing factories are inoculated with *Sacch. fibuligera*, which has the ability to hydrolyse and ferment soluble starch. The resulting mixture of oligosaccharides and reducing sugars is used to grow *C. tropicalis*, which can be used as a dietary supplement.

Although SCP may be produced from a variety of micro-organisms, fungi have a number of advantages. Fungi have been well studied and characterized and the eating of fungi (e.g. mushrooms) and products containing fungi (e.g. Roqueforti cheeses) is well accepted. In addition, fungi have a low nucleic acid content and their filamentous morphology can be manipulated to give a fibrous appearance similar in appearance and texture to meat. In recent years, fungi have been exploited for the production of SCP, which is available commercially as a human food.

Fusarium graminearum is employed for the manufacture of mycoprotein and is marketed as Quorn™ mycoprotein. The search for a good producer of mycoprotein (also referred to as SCP) commenced in 1968, when Ranks, Hovis and McDougal Ltd. began screening over 3000 soil isolates for a fungus capable of growing on starch and giving a high yield of protein. Initial work concentrated on a *Penicillium* species but an *F. graminearum* isolate (labelled A3/5) proved superior. Isolate A3/5 had an optimum growth temperature of 30 °C and a mean generation time of 3.5 hours. When grown in medium containing 100 g glucose, 54 g of fungus results, of which 45 per cent (by weight) is protein.

The fermentation is now conducted in an air-lift continuous fermenter with cycle times of approximately 3000 hours (Figure 4.7). The culture is maintained

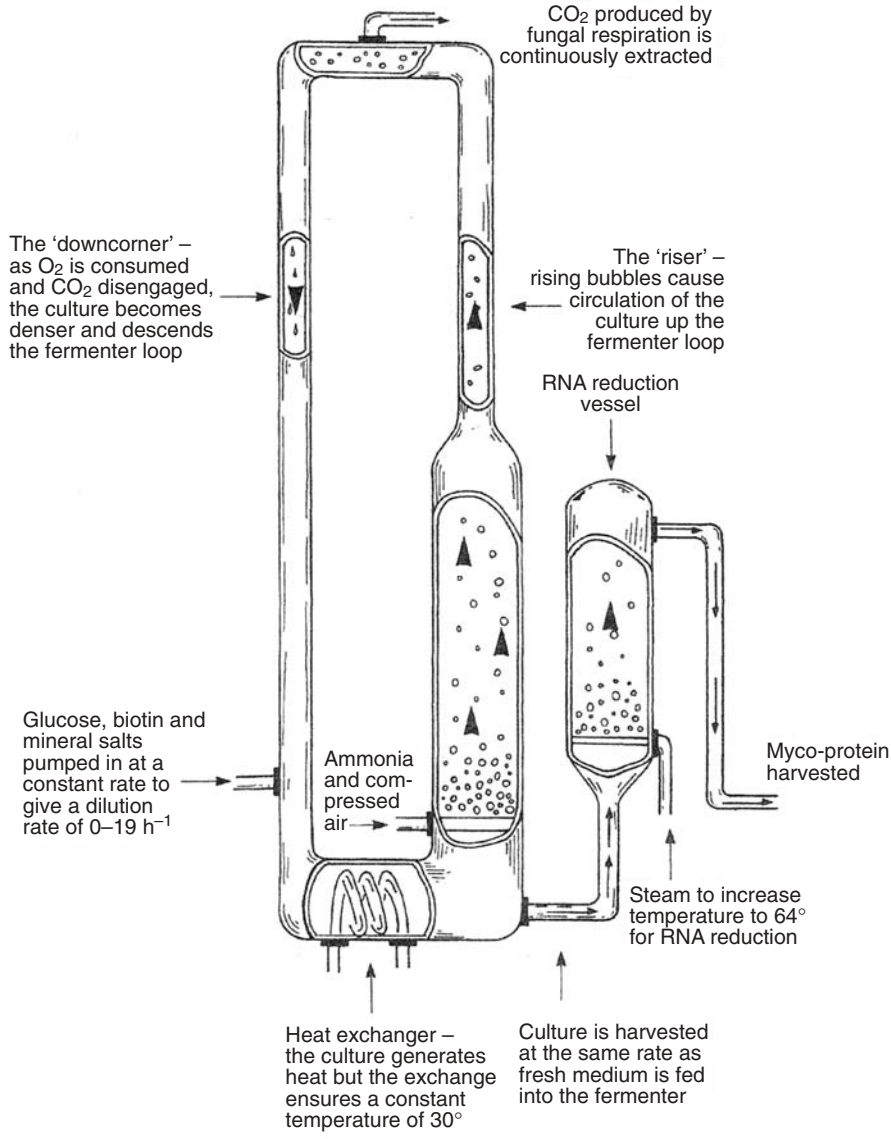
CO_2 produced by fungal respiration is continuously extracted
The 'downcorner' – as O_2 is consumed and CO_2 disengaged, the culture becomes denser and descends the fermenter loop
The 'riser' – rising bubbles cause circulation of the culture up the fermenter loop
RNA reduction vessel
Glucose, biotin and mineral salts pumped in at a constant rate to give a dilution rate of 0–19 h^{-1}
Ammonia and compressed air
Myco-protein harvested
Steam to increase temperature to 64° for RNA reduction
Heat exchanger – the culture generates heat but the exchange ensures a constant temperature of 30°
Culture is harvested at the same rate as fresh medium is fed into the fermenter

Figure 4.7 Continuous fermenter used in the production of Quorn™ mycoprotein (reproduced with permission from Trinci (1991) *Mycol. Res.* **96**(1): 1–13)

in the exponential phase of growth and the feedstock is starch. One problem associated with the long fermentation time is the appearance of a highly branched variant that arises spontaneously in the culture, which adversely affects the texture of the final product. The appearance of the variant can be minimized by limiting nutrients and regulating pH.

Fungal mycelium is harvested on a continual basis, heat treated to reduce the RNA content from 10 to 2 per cent and processed for marketing. The requirement to reduce the RNA content by thermal treatment is due to the fact that a high RNA intake can lead to a build-up of uric acid in the body, leading to deposition in the kidneys and joints. Fungal mycoprotein has a net protein utilization (NPU) of 75, compared with a value of 80 for beef and 83 for fish. The fungal mycelium can be processed to give the appearance and 'mouth-feel' of meat.

Quorn mycoprotein was launched on the market in 1985 and in 1999 over 790 tonnes were produced. It is sold in ready to eat meals and also in the form of cubes of SCP, which can be flavoured and used in home cooking in much the same way as chicken. It has the advantage of being suitable for vegetarians and those on low-calorie diets since it contains no animal ingredients and is low in cholesterol and fat. It is currently available in a range of European countries and the USA.

4.4.3 Antibiotic production by fungi

Perhaps one of the most important discoveries regarding the beneficial use of fungi for humans was the identification in 1928 by Sir Alexander Fleming that an isolate of *Penicillium notatum* produced a substance capable of killing Gram+ bacteria. This compound was subsequently identified as penicillin and was the first member of the β-lactam class of antibiotics to be discovered. These compounds function by inhibiting peptidoglycan synthesis in bacteria and their use has reduced the importance of the Gram+ bacteria as a cause of disease. Subsequent to the identification of penicillin production by *P. notatum*, a screen revealed that *Penicillium chrysogenum* was a superior producer. Following a series of mutagenic and selection procedures, the strain used in conventional fermentations is capable of producing penicillin at a rate of 7000 mg/l compared with the 3 mg/l of Fleming's *P. notatum* isolate. The type of fermentation system that is employed has also changed, so that most systems are submerged fermentations rather than solid fermentation systems. A typical penicillin fermentation yields three types of penicillin, namely F, G and V. The latter can be used directly; however, G is modified by the action of penicillin acylase to give a variety of semi-synthetic penicillins, which show resistance to the action of bacterial penicillinases, which are implicated in conferring anti-bacterial drug resistance.

The majority of antibiotics obtained from fungi are produced by fermentation and most are secondary metabolites, production of which occurs in the stationary phase and is linked to sporulation. Catabolite repression can inhibit antibiotic production and one way to avoid this is to use low levels of glucose in the fermentation medium or to obtain a mutant that is not catabolite repressed. The chemical content of the medium must be monitored since high

levels of nitrogen or phosphate (PO_4) retard antibiotic production. One problem that seriously affects the productivity of antibiotic fermentations is feed-back inhibition, where the antibiotic builds to high intracellular levels and retards production or kills the cell. One means of reversing this is to introduce low levels of the anti-fungal agent amphotericin B, which increase membrane permeability, leading to a decrease in intracellular antibiotic levels and a concomitant increase in production.

Antibiotic production can be maximized by optimizing production as a result of random mutagenesis and selection. Another approach has been to fuse or mate strains capable of producing high amounts of product with strains that are good secretors of product. Rational selection is a process where a chelating agent is introduced into the fermentation to complex all the metal ions present and consequently has a beneficial effect on antibiotic production. More recently, genetic cloning has been employed to express the genes for antibiotic production in another species – this has the possibility of producing hybrid antibiotics with potential novel targets.

4.4.4 Enzyme production by fungi

Fungi have been widely exploited as a source of industrially important enzymes for many years and the principal fungi used in this regard are members of the *Aspergillus* and *Penicillium* genera (Table 4.5). Fungi have been the organism of choice for enzyme isolation since their biology is well characterized and the ones of interest have been awarded 'generally regarded as safe' (GRAS) status.

Table 4.5 Fungal enzymes: sources and applications

Fungus	Enzyme	Application
Yeast		
S. cerevisiae	Alcohol dehydrogenase	Ethanol assay
S. cerevisiae	Invertase	Confectionery
Kluyveromyces lactis	Lactase	Dairy industry
Mould		
Aspergillus niger	β-glucanase	Brewing
Aspergillus oryzae	Protease	Meat tenderizer
A. oryzae	α-amylase	Food industry
A. niger + *A. oryzae*	Lipase	Dairy industry
Aspergillus species	Pectinase	Fruit juice clarification
Trichoderma viride	Cellulase	Dehydrated foods
Mucor miehei	Rennin	Cheese manufacture

Catalase (EC 1.11.1.6) is used in cold sterilization and has been isolated from *Aspergillus niger*, lipase (EC 31.1.3, glycerol ester hydrolase) is used as a flavour enhancer and amylase (EC 3.2.1.1, α-1,4-glucan 4-glucanohydrolase), used for malting barley or improving the quality of bread, has been obtained from a number of *Aspergillus* species. Glucose oxidase (EC 1.1.3.4, β-D-glucose: O_2 oxido-reductase) is employed in glucose assays and can be isolated from *Penicillium notatum*. Other enzymes that are obtained from filamentous fungi or yeast include cellulase (EC 3.2.1.4, β-1,4-glucan glucanohydrolase), which degrades cellulose, invertase (EC 3.2.1.26, β-D-fructofuranoside fructohydrolase), which converts sucrose to glucose and fructose, and is used in jams and confectioneries, and pectinase (EC 3.2.2.15, polygalacturonide), which is used in the clarification of wine must and fruit juices.

Traditionally, fungi cultivated for the isolation of enzymes have been grown under solid fermentation conditions, where the fungus is allowed to grow across a solid substrate in a low water environment. Typically, the substrate has been bran or other grain-based material although recently other fermentation systems such as fed-batch, submerged or continuous cycle, using a wider range of carbon sources, have been utilized. Fungi are a good source of a number of enzymes and the isolation of these enzymes from fungi has many advantages over the use of animal or plant cells as sources of enzyme. Fungi display metabolic flexibility; that is, there are many variations in their metabolic pathways compared with animal cells. They can be grown readily using simple growth media and are amenable to genetic manipulation. Strain development to enhance enzyme production or stability can be achieved using mutagenesis or by applying a selection pressure for a specific enzyme. Fungi can also be transformed using a variety of vectors to produce recombinant proteins. Fungal enzymes, like cells, may be immobilized to increase their efficacy or to maintain them in a reactor. Immobilization of enzymes increases their stability but may also alter the pH at which they demonstrate optimum activity. One of the earliest examples of enzyme immobilization was performed by Tate and Lyle, who attached invertase to charcoal, which was subsequently used to hydrolyse sucrose.

In terms of fungal enzyme fermentation, one of the main problems is the control of bioreactor temperature, particularly when large volumes are used. Another problem encountered at the industrial level is the loss of strain-specific characteristics during prolonged fermentation, which can adversely affect the production of the desired enzyme. Due to the nature of the fungal cell wall intra-cellular enzymes must be released from the cell by some form of disruption. Methods such as mechanical breakage, alkali treatment and enzyme digestion of the cell wall have all been employed to release the enzyme(s) of interest. Following release of the enzyme there is a requirement to 'clean up' the material by removing nucleic acids, membranes and solids. The enzyme may be purified by affinity purification and used in its free state or in an immobilized state.

4.4.5 Mushroom production

Mushrooms are widely consumed throughout the world and are produced in a multi-staged process. China, the USA and the Netherlands are the biggest producers of mushrooms, producing 442 000, 358 000 and 225 000 tonnes respectively. The biggest importer of fresh mushrooms is the UK (47 000 tonnes), while the Netherlands and Ireland are the two biggest exporting countries. In western Europe the most commonly consumed mushroom is the edible mushroom (*Agaricus bisporus*), but in the far East species such as *Pleurotus*, *Lentinus edodes* and *Auricularia* are the dominant cultivated mushrooms.

The growth medium for the cultivation of *A. bisporus* is compost, which consists of stable manure, straw and possibly chicken manure. The compost is spawned with a culture of *Agaricus bisporus* (edible mushroom) grown on sterilized cereal grains and cultured at 25–28 °C and high humidity for 14 days to allow the mushroom mycelium to colonize the compost. The chitin-rich mycelium consists of bunches of septate hyphae, which digest food externally and absorb digested material as the fungus grows throughout the compost. After this period the compost is 'cased' with a layer of neutralized peat, which has the effect of stimulating the formation of large primordia, which will later develop into mushrooms. The exact function of casing is unclear, but it may provide biotic, chemical and/or physical factors that trigger mushroom formation. Under ideal conditions and in the absence of overt disease, flushes or crops appear at weekly intervals until the nutrients in the compost are depleted.

Fungal diseases of mushrooms

The conditions under which mushrooms are produced provide an ideal environment for the growth of a wide range of potentially damaging fungi. The fungi that affect mushroom crops can be divided into two broad classes: the 'weed moulds', which typically grow in the compost and compete with the mushroom mycelium for nutrients, water and space, and the fungal pathogens (or mycopathogens), which directly attack the mushroom. Weed moulds include such species as *Trichoderma*, *Chrysporium* and *Coprinus* and may affect the actual growth of the mushroom, but their impact on crop yield may be variable. Some species of *Trichoderma* (often referred to as green mould) grow on the casing while others, such as *Chrysosporium*, grow at the casing – compost interface and inhibit the growth of the mushroom mycelium.

The principal fungal pathogens of mushrooms are *Verticillium fungicola*, *Mycogone perniciosa* and *Hypomyces rosellus*. Each disease is highly destructive and the spores of each fungus are dispersed by air currents, water splashes and human contact. Dry bubble is caused by *Verticillium fungicola* and is characterized by a swelling of the mushroom stipe and the appearance of blue-grey

spots on the cap, from which a grey coloured mycelium may develop. Wet bubble is caused by *Mycogone pernicosa* and infects cultivated mushrooms and probably wild mushrooms and toadstools also. Under humid conditions infected mushroom stipes swell and the caps are small and misshapen. Amber-coloured droplets appear over the surface of the mushroom, which subsequently collapses into a wet bubble-like decaying mass. Cobweb disease of mushrooms is caused by *Hypomyces rosellus*, which colonizes the mushroom and also grows through and over the surface of the casing soil. Infected mushrooms become engulfed by the growth of the cobweb-like mycelium and the gills fail to develop.

The above pathogens are responsible for approximately 90 per cent of all fungal diseases of mushrooms and cause considerable economic loss to mushroom growers. Conventional control measures involve the use of fungicides and the adherence to strict hygiene standards. Under ideal conditions a grower would expect four crops from a batch of compost but due to the presence of the above mycopathogens the usual harvest is two or three flushes.

4.5 Genetic manipulation of fungi

Fungi may be genetically manipulated in a number of ways to increase the production of a particular product, to raise the tolerance to a specific factor or to render them capable of expressing and secreting a foreign, recombinant protein. The reasons for genetically manipulating a brewing yeast are many and varied but may include some of those listed in Table 4.3. Genetic manipulation of fungi may be achieved in a number of ways. Mutagenesis has been attempted using a number of approaches. Chemical mutagenesis may be performed using agents such as sodium nitrite or MNNG and usually results in a single nucleotide substitution in the target genome. Ultraviolet light induces errors in DNA replication, while ionizing radiation, as a mutagen, induces deletions and translocations. Mutagenesis can be employed to isolate a mutant with the desired characteristic(s) but should be used with caution since, in addition to the known mutation, other non-specific mutations may be introduced, which can be deleterious. A good example of the ability of mutagenesis to increase yield was evident in penicillin production from *Penicillium chrysogenum*, which was increased from 550 mg/l to 7000 mg/l following a programme of UV and nitrogen mustard mutagenesis along with selection of spontaneous mutants.

The process of 'rare mating' has been employed with brewing strains of *S. cerevisiae*. In this process high numbers (10^8) of complimentary non-mating cultures of yeast are mixed and occasionally some spontaneous mating occurs to give hybrids that may express improved fermentation characteristics. Another characteristics often used with brewing strains is the process of single chromosome transfer, which involves the transfer of a chromosome from one strain to another, which may result in an altered phenotype in the target yeast.

The ability to enzymatically remove fungal cell walls and produce a protoplast has been exploited to generate strains capable of enhanced ethanol production or the metabolism of a novel combinations of carbohydrates. Once protoplasts are formed they may be fused by incubation in polyethylene glycol and calcium ions, which induce membrane breakdown. Selection of the resulting fusants ensures the isolation of hybrids combining characteristics of both parents. Protoplast fusion has been particularly successful in overcoming sexual incompatibility barriers that exist between fungal species. Protoplast fusion has been utilized to generate strains of *S. cerevisiae* that produce glucoamylase, overproduce the amino acid methionine and ferment xylose to ethanol.

Fungal cell transformation is also possible and was first demonstrated using protoplasts of *S. cerevisiae* by Hinnen, Hicks and Fink in 1978. Cells may be made competent for the uptake of DNA by conversion to protoplasts, by treatment with lithium acetate or using micro-projectiles coated with the relevant DNA. The ability to transform yeast has opened the way to introducing many characteristics and constructing strains with novel characteristics. Many of these strains have been assessed for their economic potential.

In addition to their well established uses in the production of alcoholic beverages, bread, enzymes and antibiotics, fungi are now being exploited as expression systems for the production of foreign proteins. The *S. cerevisiae* expression system for producing recombinant protein has a number of advantages including producing yields of purified secreted protein in the region of 20 mg/l. *S. cerevisiae* has been employed for the production of small hepatitis B surface proteins for use in recombinant hepatitis B vaccines and in the production of several recombinant malarial proteins, again for use in vaccines. Anti-malarial vaccine components produced by *S. cerevisiae* are in clinical trials and may eventually be used clinically. *S. cerevisiae* has also been employed to produce the interferon class of cytokines, which are important in the treatment of diseases such as AIDS-associated Kaposis' sarcoma and to prevent re-occurrence of infections in patients suffering from chronic granulomatous disease. Currently, almost half of the world's diabetic patients use insulin produced by *S. cerevisiae*.

Yeast are a popular choice for foreign gene expression as they have traditionally been classed as GRAS (generally regarded as safe), are easy to cultivate and are physically robust (Table 4.6). From a genetic point of view, *S. cerevisiae* is an eukaryotic organism, possesses a small genome and often contains natural plasmids (e.g. 2μM circle), which can be utilized for transformation. In terms of their use as a molecular biological tool, yeast strains can be transformed easily, are generally good secretors of protein and splice introns from animal genes and their RNA polymerase recognizes animal promoters. A number of vectors are utilized for transforming yeast cells and include integrative plasmids (e.g. Yip), independently replicating plasmids (e.g. Yrp, Yep) and specialized plasmids (e.g. yeast killer plasmid, yeast expression plasmid). Once a yeast has been transformed with a plasmid containing the gene of interest, the gene is transcribed and translated (Table 4.7). Post-translation modification (e.g. gly-

Table 4.6 Advantages and disadvantages of using yeast for foreign gene expression

Advantages
Historical
Generally regarded as safe (GRAS)
Widely used
Technological
Simple to culture
Well-characterized fermentation
Grow on cheap carbon sources
Genetic
Eukaryotic
Small genome
Possess natural plasmids (2μM)
Molecular biological
Efficient transformation systems
Protein secretion
Splice introns in animal genes
Disadvantages
Plasmid instability leads to loss of characteristics
Hyperglycosylation
May not be suitable for certain proteins
Need to identify optimum yeast expression system

Table 4.7 Steps in heterologous gene expression in yeast

Gene isolation: isolate gene of interest.
Cloning: insert gene into expression vector.
Transformation: introduce vector into yeast cell.
Selection: identify transformants.
Expression: transcription and translation of protein.
Modification: post-translational chemical modification (glycosylation).
Signalling: secretion of signal peptide.
Secretion: export of protein from cell.

cosylation) occurs and secretion involves export of the protein of interest from the cell. Due to the fact that *S. cerevisiae* is not a prolific secretor of foreign protein, superior secretors such as the yeasts *Candida maltosa*, *Yarrowia lipolytica* and *Pichia* species have become more routinely employed in recent years. Some disadvantages are also associated with the use of yeast for the expression of recombinant genes. The ability to produce a recombinant protein at the laboratory scale often does not translate to the pilot plant stage due to loss of the

relevant plasmid during scale-up. In addition, some proteins are hyperglycosylated by fungi, which can alter their immungenicity and provoke adverse effects upon administration.

4.6 Conclusion

Fungi have been utilized for thousands of years for the production of various foods and beverages. While these applications are still important, fungi are now being used in novel ways for the production of SCP, antibiotics and enzymes and as expression systems for the production and secretion of foreign proteins. Consequently, the continued use of fungi on a large scale by humans is guaranteed.

4.7 Further reading

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4.8 Revision questions

Q 4.1 Outline the main fermentation systems used commercially with fungi. Give examples of a product generated by each system.

Q 4.2 Describe the four methods for immobilizing yeast cells.

Q 4.3 What factors affect the productivity of a fermentation?

Q 4.4 Describe how recent advances in brewing technology could impact on the type of beer consumed in the future.

Q 4.5 Describe the processes involved in the production of Quorn mycoprotein.

Q 4.6 What factors affect the synthesis of antibiotics by fungi?

Q 4.7 What fermentation system is used to produce fungal enzymes?

Q 4.8 Outline the stages in the cultivation of *Agaricus bisporus*.

Q 4.9 What is meant by the term 'rare mating'?

Q 4.10 Why are yeast good candidates for the expression of foreign genes?

5

Antibiotics, Enzymes and Chemical Commodities from Fungi

Richard A. Murphy and Karina A. Horgan

5.1 Introduction

The economic significance of fungal biotechnology cannot be understated; indeed, as this chapter will outline, fungi have been exploited to yield a range of important products, some of which have proved invaluable to mankind. Since the time of the Pharaohs, fungi have been utilized for simple food processing; however, the last century has seen the development of fungal biotechnology for the subsequent production of valuable commodities such as antibiotics, enzymes, vitamins, pharmaceutical compounds, fungicides, plant growth regulators, hormones and proteins. As we move into the 21st century, this list will expand further, but it is beyond the scope of this chapter to fully appreciate the enormous benefits and economic impact of fungi in the area of biotechnology. Instead, we will concentrate on a number of the more economically significant production processes, which have been developed through the utilization of fungi. The diverse nature of some of the economically important products produced by fungi is demonstrated in Table 5.1.

5.2 Fungal metabolism

A common link between all fungi is their heterotrophic nature: they cannot manufacture their own food and depend on the organic material in other

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Table 5.1 Fungal products of economic importance

Class of product	Typical example	Industrial/commercial application	Common production organism
Enzymes	Amylase	Starch processing	*Aspergillus niger*
		Fermentation application	*Rhizopus oryzae*
	Cellulase	Animal feed industry	*Trichoderma longibrachiatum*
		Brewing	
	Protease	Meat/leather industry	*Aspergillus oryzae*
		Cheese manufacture	*Rhizopus oligosporus*
Organic acid	Citric acid	Soft drinks industry	*Aspergillus niger*
	Itaconic acid	Chemical industry	*Candida/Rhodoturula*
	Malic acid	Beverage/food industry	*Candida*
	Fumaric acid	Food industry	*Candida*
Vitamins	Riboflavin	Health industry	*Candida*
	Pyridoxine	Health industry	*Pichia*
	D-erythro-ascorbic acid	Health industry	*Candida*
Antibiotics	Penicillin	Human/animal health	*Penicillium chrysogenum*
	Cephalosporin	Human/animal health	*Cephalosporium acremonium*
Fatty acids	Stearic	Food industry	*Cryptococcus*
	Dicarboxylic	Chemical industry	*Candida*
Alcohol	Industrial alcohol	Fuel industry	*Saccharomyces*
	Beverage alcohol	Beverage industry	*Saccharomyces*
Pharmaceuticals	Lovastatin	Human health	*Monascus ruber*
	Cyclosporin	Human health	*Tolypocladium inflatum*
Amino acids	Lysine	Health industry	*Saccharomyces*
	Tryptophan	Health industry	*Hansenula*
	Phenylalanine	Health industry	*Rhodoturula*
Recombinant proteins	Insulin	Treatment of diabetes	*Saccharomyces cerevisiae*
	Phytase	Phosphate liberation	*Aspergillus niger*
	Hepatitis B surface antigen	Vaccine preparation	*Saccharomyces cerevisiae*

organisms for their survival. In a broad sense, however, it is possible to ascribe fungi into two main groups depending on how they obtain and assimilate nutrients. One group, the parasitic and mutualistic symbionts, obtains its nutrients in an effective manner from living organisms. The second group, saprotrophs, has the ability to convert organic matter from dead organisms into the essential nutrients required to support growth. It is this second group that we are particularly interested in, as this group of organisms gives rise to the production of the main bulk of the commodities commonly associated with fungi. However, regardless of this division, within the fungal life cycle one can clearly delineate the production of certain products or metabolites into two phases, namely primary and secondary metabolism.

Primary metabolites are those that are essential for growth to occur and include proteins, carbohydrates, nucleic acids and lipids. Indeed, the precursors of these primary products must be synthesized if they cannot be obtained from the growth medium. These primary metabolites have essential and obvious roles to play in the growth of the fungus. Typically, primary metabolites are associated with the rapid initial growth phase of the organism and maximal production occurs near the end of this phase. Once the fungus enters the stationary phase of growth, however, primary metabolites may be further metabolized. Examples of primary metabolites produced in abundance include enzymes, fats, alcohol and organic acids. Economically speaking, primary metabolites are easily exploited as the biochemical pathways involved in their production are widespread throughout the fungal kingdom. This allows for the rapid screening of classes of fungi for such products and the rapid development of production processes for their utilization.

Primary metabolic processes have also been extensively usurped through the use of recombinant DNA technologies, to the extent that heterologous proteins can be routinely produced by the host fungus as part of its primary metabolic phase. In contrast to the primary metabolites, secondary metabolites are not essential for vegetative growth and indeed may have little or no primary function within the organism. Secondary metabolites are produced when the organism enters the stationary phase, once the initial phase of rapid growth has declined. The metabolites produced in this phase are often associated with differentiation and sporulation and can have profound biological activities, which in some instances have been exploited economically. A number of distinct differences are apparent between primary and secondary metabolites. In the first instance they have been shown to possess an enormous variety of biosynthetic origins and structures that are not, in general, found among the primary metabolites. Second, their occurrence tends to be restricted to a small number of organisms and indeed can vary between individual strains of the same species. Finally, their production is characterized by the generation of groups of closely related compounds, which may have very different biological properties.

Important examples of secondary metabolites include medically important compounds such as antibiotics, statins, cyclosporins and ergot alkaloids. Agri-

Table 5.2 Example of primary and secondary metabolites

	Example	Production organism
Primary metabolites	Enzymes	*Aspergillus* sp.
	Industrial alcohol	*Saccharomyces cerevisiae*
	Organic acids	*Aspergillus/Candida*
	Fats	*Candida*
	Polymers	*Yarrowia*
Secondary metabolites	*Antibiotics*:	
	penicillin	*Penicillium*
	fusidic acid	*Fusidium coccineum*
	Cholesterol lowering agents:	
	lovastatin	*Monascus ruber*
	mevastatin	*Penecillium citrinum*
	Immunosuppressing drugs:	
	cyclosporin A	*Tolypocladium inflatum*
	Plant hormones:	
	giberellic acid	*Gibberella fujikuroi*

culturally important secondary metabolites include strobilirubin, an antifungal compound, and plant hormones such as giberellic acid. Table 5.2 outlines some of the more commonly found primary and secondary metabolites and the organisms from which they have been commercially exploited.

Fungal biotechnology has developed, to allow the utilization of the metabolic processes inherent to the organisms, in a commercially viable manner. In this chapter we will detail a number of the more important commercial commodities produced by fungi and outline the production processes for them.

5.3 Antibiotic production

5.3.1 Overview

The most studied secondary metabolites are a class of compounds known as antibiotics. These low-molecular-mass compounds are so called because at low concentrations they inhibit the growth of other micro-organisms. While many thousands of antibiotics have been discovered, their use has been limited to perhaps 60 at most due to the toxic properties they exhibit towards humans. Clinically speaking the majority of antibiotics are produced by the Actinomycetes, a bacterial order, and will not be dealt with here. Whilst several fungal genera produce antibiotics, only two do so to a commercially viable extent, *Aspergillus* and *Penicillium*. The β-lactams, of which penicillin is the most famous, not least because of its fortuitous discovery by Fleming in 1928, com-

prise a very large group of antibiotics and include both the cephalosporins and penicillins. In 2000 the estimated world market for antibiotics was $28 billion, which underlies the importance both medically and economically of these metabolites.

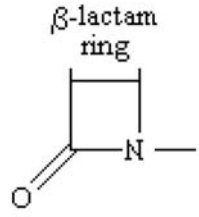
The word penicillin can be regarded as a generic term used to describe a large group of natural and semi-synthetic antibiotics that differ only by the structure of the side chains on the core aminopenicillanic acid ring. As a rule the basic penicillin molecule consists of a β-lactam ring, a five-membered thiazolidine ring and a side chain. β-lactams with non-polar side chains such as phenylacetate and phenoxyacetate are hydrophobic in nature and include penicillin G (benzylpenicillin) and penicillin V (methylpenicillin). The non-polar penicillins are synthesized only by filamentous fungi.

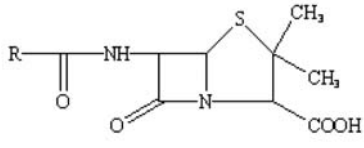
At their core they all possess a β-lactam (four-atom cyclic amide) ring on which side-chain substitutions and differences give rise to a series of antibiotics each with differing anti-bacterial activity. In addition to the so-called classical β-lactams, semi-synthetic varieties can be manufactured by the removal of the naturally occurring side chains and the subsequent chemical derivitization of the core β-lactam ring. Figure 5.1 illustrates the core β-lactam ring and the basic structures of penicillin and cephalosporin.

Gram positive bacteria possess on the outer aspect of the cell wall a layer that is composed of characteristic groupings of proteins and carbohydrates that comprise the antigenic determinants responsible for generating an immune response. Inside this outermost layer there is a polymeric structural layer known as peptidoglycan which is composed of repeating units of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). Associated with this cell wall structure are a number of proteins known as penicillin-binding proteins (PBP); the function of some is unclear. During cell wall biosynthesis, a cross-linking process occurs, whereby peptidoglycan strands become linked, leading to the structural stability of the wall. It is this cross-linking that is extremely sensitive to β-lactam antibiotics. For instance, various penicillins bind to the PBPs through their different side-chains, leading to a variety of effects. Reaction with PBP-1 (a transpeptidase) produces cell lysis, while binding to PBP-2 (also a transpeptidase) leads to the generation of oval cells, which are unable to replicate. Cephalosporins act in a very similar fashion to the penicillins and are also able to react with the PBPs by forming covalent bonds, thus leading to cellular lysis.

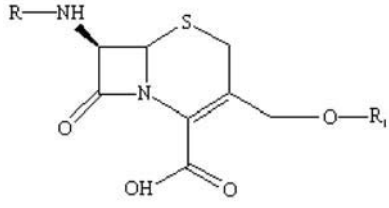
Gram negative cells have a more complex cell wall structure and usually contain an outer membrane and a complex periplasm containing lipopolysaccharides. Whilst the gram negative cell wall also contains a peptidoglycan layer, it is not as extensive as that of gram positive bacteria but is sensitive to β-lactam antibiotics due to the presence of PBPs.

Penicillins with polar side chains, such as D-α-aminoadipate, include penicillin N and possess hydrophilic characteristics. They are more widely synthesized by a range of micro-organisms, including fungi, actinomycetes and unicellular bacteria.





Basic penicillin structure

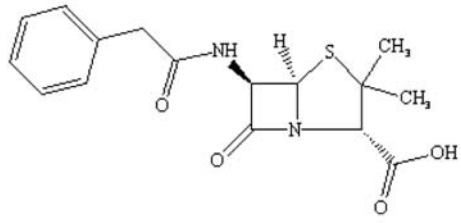


Basic cephalosporin structure

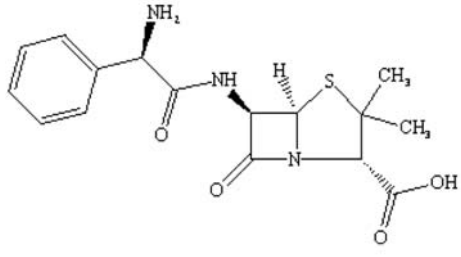
Figure 5.1 Structures of the core β-lactam ring and β-lactam antibiotics

The production of semi-synthetic penicillins is quite easy and involves the removal of the side-chain from a naturally occurring penicillin and its subsequent replacement with a different side-chain to yield a novel β-lactam derivative. Examples of semi-synthetic varieties include methicillin and ampicillin. Figure 5.2 illustrates the structure of a natural and a semi-synthetic penicillin.

One serious problem with penicillins relates to the highly reactive nature of the β-lactam ring, which can result in their being susceptible to a variety of degradation processes. Factors that can affect their stability include their reactivity with hydroxide ions, which can result in the formation of inactive penicilloic acid, or their acid-sensitive nature, which can lead to their degradation at low pH. Acid sensitivity can be overcome clinically by use of the compounds in a buffering solution. A more serious limitation to their use is their susceptibility to a group of enzymes known as penicillinases, which are produced by bacteria and can result in the development of antibiotic resistance. The most common of these enzymes is β-lactamase, which cleaves the β-lactam ring and thus inactivates the antibiotic. A variety of acylases have also been identified,



Penicillin G (natural)

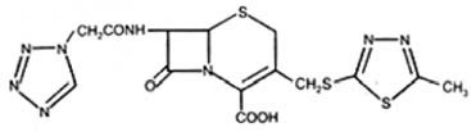


Ampicillin (semi-synthetic)

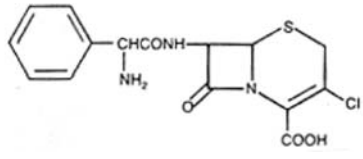
Figure 5.2 Natural and semi-synthetic penicillins

whose mode of action is to cleave the acylamino side-chain of the antibiotic, thus rendering it inactive. To combat these enzymes a number of compounds such as clavulinic acid or the sublactams have been developed, which when given in combination with the susceptible antibiotic result in the permanent inactivation of the antibiotic-degrading enzymes. Of more importance, however, has been the development of the semi-synthetic penicillins, many of which are resistant to β-lactamase and other penicillinases. For instance, methicillin is completely resistant to these enzymes, though it does have the disadvantage that it is less effective. Almost all β-lactamase-resistant penicillins are less potent than the parent molecules.

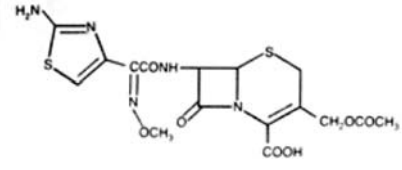
Cephalosporins are very structurally and chemically closely related to the penicillins, however, unlike the penicillins their use was limited for a long period until a clinically useful agent was found. Cephalasporin C is regarded as the prototypical cephalosporin and following its structural elucidation it was found to be a β-lactam with a six-membered dihydrothiazine ring instead of the five-membered thiazolidine ring characteristic of the penicillins. Chemical removal of the side chain of cephalosporin C results in the generation of 7-aminocephalosporonic acid (7-ACA), which can be used as a synthetic starting point for most of the cephalosporins available today. Indeed, it is more economically feasible to produce 7-ACA from penicillin G by a series of synthesis reactions rather than to incur the prohibitive costs of fermentation to produce the antibiotic.



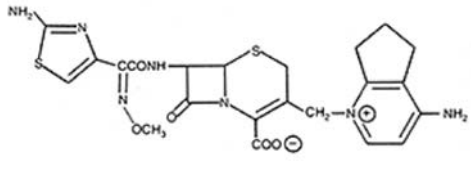
Cefazolin (1st generation)



Cefachlor (2nd generation)



Cefotaxime (3rd generation)



Cefpirome (4th generation)

Figure 5.3 Cephalosporin structures

One notable feature of the structure of the cephalosporins is their reduced chemical reactivity relative to the penicillins. However, some β-lactamases are more efficient at cleaving cephalosporins than penicillins and this has led to the development of so-called second-, third- and fourth-generation cephalosporins. These compounds all differ in their antimicrobial properties, susceptibility to microbial resistance, absorption, metabolism and side-effects. Examples of first-generation cephalosporins include cepalothin and cephazolin, second-generation cephalosporins include cefamandole and cefachlor, third-generation cephalosporins include cefotaxime and cefixime and fourth-generation examples include cefapime. The structures of the so-called first-, second-, third- and fourth-generation cephalosporins are illustrated in Figure 5.3.

5.3.2 Fungal production cycles

At a cellular level, the production pathways for the cephalosporins and penicillins share some similarities and indeed the first two steps are common to both classes of antibiotic. Initially, a tri-peptide known as ACV is formed from the amino acids L-cysteine, L-α-aminoadipic acid and L-valine. This key intermediate is then converted to isopenicillin N (IPN) by the enzyme IPN synthase. It is this intermediate that gives rise to both the penicillins and the cephalosporins. In the case of penicillin formation, IPN is hydrolysed to 6-amino penicillanic acid (6-APA), which can be used subsequently to give rise to specific penicillins. Alternatively, in the formation of the cephalosporins, IPN is epimerized to penicillin N, which is further reacted enzymatically to yield deoxycephalosporin C. This last molecule can undergo further modification to give rise to cephalosporin C and cephamycin C.

As discussed earlier, the generation of semi-synthetic varieties of penicillins and cephalosporins is a simple process. By reacting the core penicillin compound 6-APA with a variety of organic acids, numerous penicillins can be formed. Indeed, the production of 6-APA is now carried out through the removal of the side-chain from penicillin G and then reacted to yield a range of antibiotics. Similarly, the removal of the side-chain from cephalosporin C to yield 7-amino cephalosporonic acid (7-ACA), can lead to the generation of numerous cephalosporins through the reaction of this compound with a variety of acids.

5.3.3 Industrial production of antibiotics

Industrially speaking, penicillin production is a relatively inefficient process, where it is estimated that only 10 per cent of the carbon source utilized in the fermentation ends up as antibiotic. Production of β-lactam antibiotics occurs best under conditions of carbon, nitrogen and phosphorus limitation and at low growth rates. Each manufacturer uses a different production process, the details of which are closely guarded. Overall though, the basics of the production process are similar in nature. Production starts with the inoculation of a primary culture from a preserved culture stock. Typically the culture stock can be in the form of lyophilized spores or spores preserved in liquid nitrogen.

Primary culturing can utilize either agar slants or liquid culture, with agar slants being the most common. The primary culture is used to inoculate a secondary culture, which in the case of antibiotic production is aimed at the generation of spores. Secondary culturing can take place in agar-coated bottles or on particulate material, both of which result in the generation of a large quantity of spores.

A spore suspension prepared from the secondary culture is subsequently used to inoculate liquid media as part of an inoculum build-up process. It should be

pointed out that stringent aseptic techniques are used throughout the process to prevent the contamination of the antibiotic producing culture with a more robust micro-organism. Practically speaking, industrial strains of antibiotic-producing fungi are less robust than naturally occurring fungi due to the aggressive mutation and selection pressures placed on them when they were originally isolated.

Depending on the size of the process, the scale-up procedure can have as many as three or four stages. Typically, the initial seed culture produced from the secondary spore suspension is less than 10 l. Following a defined period of growth this can be used to inoculate a culture of less than 20 000 l, which in the final stages of the production cycle will serve to start a culture of up to 300 000 l. One important point is the nature of the product that the production cycle is centred upon. Antibiotics are secondary metabolites, and in order to obtain the maximum productivity from the final stage culture it is necessary to ensure that growth of the organism is limited and the organism enters its secondary metabolism phase. This is usually achieved by designing the growth medium to ensure that a key nutrient becomes limiting at the right time to effect the change in metabolism necessary for antibiotic production. In the case of penicillin production this is usually achieved by limiting the supply of glucose.

At the end of the fermentation it is necessary to separate the antibiotic material from the fungal mycelia, medium constituents and any other metabolites produced during the process. This is known as downstream processing, and the types of step involved will depend on the antibiotic in production and also on the production process. Typically it will involve some form of centrifugation or filtration to remove the fungal biomass and additional steps such as solvent extraction, ultrafiltration, chromatography and drying to produce a relatively pure antibiotic, which can then be used for the manufacture of pharmaceutical preparations. It is estimated that over 10 000 tonnes of penicillin G are produced by fermentation each year.

5.3.4 Additional fungal antibiotics

Fungi also produce a number of other antibiotics, which are structurally unrelated to the β-lactams. Griseofulvin, a natural organic compound containing chlorine, is produced by *Penicillium griseofulvin*. This compound is interesting as it inhibits the growth of fungi by preventing the assembly of fungal microtubules and thus mitosis. Another unrelated antibiotic is the steroidal compound fusidic acid, which is produced by *Fusidium coccineum*. This antibiotic is active against gram-positive bacteria and has clinical use against β-lactam-resistant strains of bacteria.

5.4 Pharmacologically active products

5.4.1 Overview

In addition to antibiotics, fungi, as illustrated in Table 5.2, produce a range of other secondary metabolites. Some of these compounds are very significant in terms of their medical importance, including cyclosporin A and a group of compounds with cholesterol-lowering properties known as the statins. Other compounds that will be discussed include the alkaloids and the gibberellins.

5.4.2 Cyclosporin A

Immunosuppressive drugs have transformed modern transplant surgery by vastly reducing the incidence of organ rejection. The discovery and exploitation of the powerful immunosuppressant cyclosporin A has relied almost completely on fungal biotechnology. Indeed, attempts at chemically synthesizing the drug have served to illustrate the complexity of fungal secondary metabolism. Cyclosporin A is produced by the fungus *Tolypocladium inflatum* and was initially isolated from a Norwegian soil sample. The compound inhibits the production of interleukin-2 by T-lymphocytes and in so doing inhibits any potential immune response stimulated by antigens produced against transplanted organs. Cyclosporin A has also found use in the treatment of medical conditions such as psoriasis and eczema, due to the role of interleukin-2 in mediating inflammatory responses.

Cyclosporin A is a heavily methylated cyclic peptide. In a similar fashion to other secondary metabolites, a range of over 25 cyclosporin analogues is produced by *T. inflatum*, and while 17 have antifungal activity only two are immunosuppressants. Following a series of strain improvements using mutagenesis and culture optimizations, gram per litre yields have been achieved under optimized fermentation conditions. Despite the best efforts at chemical synthesis of the drug, production of cyclosporin A is still only economically feasible by natural means.

5.4.3 Statins

The statins are a group of compounds that act as potent competitive inhibitors of 3-HMG-CoA reductase, a key enzyme in the biosynthesis of cholesterol. These organic acids interact with the enzyme through their acidic side-groups and in so doing reduce plasma cholesterol levels. The most important statins commercially are the mevinic acids, with the most notable being lovastatin from *Monascus ruber* and mevastatin from *Penicillium citrinum*. Both mevastatin and lovastatin can be converted into the compounds ML-236A and monacolin J

respectively by chemical means or by microbial transformation. Each of these compounds differs in affinity for 3-HMG-CoA-reductase and thus in effectiveness. Research and development over the last two decades has shown that a number of fungi produce a range of similar compounds with cholesterol-lowering abilities. One interesting aspect of their commercial production lies in the ability to produce these compounds by liquid fermentation (e.g. lovastatin from *Aspergillus terreus*) or by using solid-state fermentation, the so-called Koji process (e.g. lovastatin from *Monascus rubber*).

5.4.4 Alkaloids

Members of the genus *Claviceps*, a parasitic fungus that grows on a wide variety of grains, synthesize numerous secondary metabolites known as alkaloids. These compounds are produced in the sclerotia of the fungus, the resting structure with which the fungus ensures its survival over winter. Many of these alkaloids are pharmaceutically important and can act in a variety of ways but they particularly affect the central nervous system, causing hallucinations or convulsions. The common core of these compounds is the tetracyclic alkaloid ring structure (ergoline nucleus) in which the nitrogen atom at position 6 is usually methylated. This core structure is derived from tryptophan and mevalonic acid and can be modified with varying degrees of complexity to give rise to a multitude of alkaloids, each differing in potency and toxicity. These derivatives can then be used in the chemical synthesis of additional pharmacologically active compounds. For instance, lysergic acid diethylamide (LSD) is synthetically derived from lysergic acid, an alkaloid produced by *Claviceps paspali*. Medically speaking, many of these alkaloids are important due to their negative effects; indeed, alkaloid-contaminated bread caused numerous outbreaks of ergotism until the 18th century. There is also evidence to suggest that the Salem witch trials were brought about following outbreaks of ergotism.

Medically useful alkaloids have, however, been isolated and the most useful of these are the alkaloids ergotmetrine and its methylated derivative methyl ergometrine. Both of these compounds stimulate contractions of uterine smooth muscle and can also be used as vasoconstrictors to control excessive bleeding after childbirth.

5.4.5 Gibberellins

The gibberellins are a group of diterpenoid compounds containing 19 or 20 carbon atoms and are capable of promoting numerous developmental processes in plants. Examples of effects that may be mediated by these compounds include the induction of bolting, production of hydrolytic enzymes and stimulation of both cell elongation and cell division. These secondary metabolites are biosyn-

thesized from mevalonic acid by *Gibberella fujikora*, though they have also been isolated from *Sphaceloma manihoticola*, *Neurospora crassa*, *Rhizobium phaseoli* and *Azospirillium lipoferrum*. They have numerous agricultural applications, including their use in malting, fruit ripening and improving fruit set and size.

5.5 Enzymes

5.5.1 Overview

In contrast to the secondary metabolites we have discussed, primary metabolites such as enzymes have clearly defined roles in the fungal life cycle. In this instance, the production and secretion of enzymes serve as facilitators, enabling the fungus to obtain essential nutrients for its growth and reproduction. However, by their very nature it has been possible to utilize these enzymes for numerous industrial processes. The industrial uses of enzymes are numerous and we will only concentrate on those of greatest economic significance. Some of the major fungal enzymes produced on an industrial scale, their roles and the producer organisms are listed in Table 5.3.

Table 5.3 Fungal enzymes of industrial importance

Enzyme	Application	Source organism
Amylase	Starch processing	*Aspergillus niger*
Cellulase	Cellulose and hemi-cellulose modification	*Trichoderma longibrachiatum*
Protease	Protein hydrolysis	*Aspergillus oryzae* *Rhizopus oligosporus*
Lipase	Vegetable oil processing	*Rhizopus oryzae*
Phytase	Phosphorus release from phytic acid	*Aspergillus niger*
Invertase		
Glucoamylase	Soluble starch processing	*Aspergillus niger*
α-amylase	Production of high-maltose syrup	*Aspergillus niger*
Rennet	Cheese manufacture	*Mucor miehie*
Pectinase	Soft-drink manufacture	*Aspergillus niger*
Invertase	Confectionery	*Saccharomyces cerevisiae*
Lactase	Dairy	*Kluyveromyces*
Raffinase	Food processing	*Saccharomyces cerevisiae*
Xylanase	Lignin degradation	*Trichoderma reesei*

5.5.2 Applications of enzymes

Proteases have been utilized in food processing for centuries and their use today is very widespread, particularly those with acidic pH optima. The biggest application for fungal protease is in cheese manufacture where the enzyme rennet is used instead of the traditional preparation, calf chymosin, to hydrolyse a specific peptide linkage in the κ-casein protein present in milk. This hydrolysis allows coagulation of the milk protein into curds, which can then be compressed and turned into cheese. The reaction itself is dependent on the presence of Ca^{2+} ions and also on the reaction temperature, thus leading to easy control of the process. The main organism used in the production of rennet is *Rhizomucor miehie*, although recombinant strains of *Aspergillus* have been developed that produce gram quantity yields of the *R. miehie* gene product. These recombinant preparations have been shown to have more favourable properties than the non-recombinant preparations. Proteases are widely used in the production of beer, where they are used to remove protein 'hazes'. In general, proteolysis increases the solubility of proteins at their isoelectric points, a phenomenon that can be exploited in haze reduction. Proteases are also used extensively in the baking industry and can be used for wheat-gluten modification in biscuit preparation. Hydrolysis of soy protein using fungal protease can increase both the usage range and value of this inexpensive protein source. Hydrolysed soy proteins can be added to cured meats or to impart flavour characteristics to soft drinks.

Starch is a polymeric structure made up entirely of glucose residues in either of two forms, α-amylose and amylopectin. In contrast to protein degradation, efficient starch hydrolysis relies on the combined actions of both bacterial and fungal enzymes. In the first instance, a starch slurry is treated with bacterial α-amylase at very high temperatures. This enzyme will cleave the α-1,4 glucosidic links within the starch molecule and results in the production of gelatinized starch, which can be further enyzmatically modified to produce a range of sugars. For example, so-called high-glucose syrup can be prepared by treating the liquefied starch with a fungal glucoamylase. This exoenzyme enzyme cleaves both the α-1,4 and α-1,6 glucosidic linkages from the ends of the polymer chains. The resultant preparation typically contain more than 97 per cent glucose, with the remainder made of maltose and higher oligosaccharide. Alternatively, so-called high-maltose syrups can be prepared by treating the liquefied starch with a fungal α-amylase. This enzyme cleaves only the α-1,4 linkages and produces a syrup containing more than 50 per cent maltose, with the remainder as higher oligosaccharides. Fungal α-amylase has also found use in the baking industry, where it is used to promote adequate gas production and starch modification during fermentation. Fungal enzymes are used in preference to bacterial enzymes due to their heat-labile nature, resulting in their rapid denaturation during baking, thus making their use easier to control.

At first glance the complete hydrolysis of cellulose, a homopolymeric β-1,4 glucose molecule, appears relatively easy. However, in nature cellulose rarely exists in pure form and is in fact intimately associated with roughly equal quan-

tities of lignin and pentosans. A combination of enzymes is therefore required to degrade this abundant structural polysaccharide and in practice the complete degradation of cellulose to glucose is prohibited by cost constraints. Typically speaking, most commercial cellulase preparations are mixtures of the following enzymes: endo-1,4-D β-glucanase (EC 3.2.1.4), glucan 1,4 β-glucosidase (EC 3.2.1.74) and cellulose 1,4 β-cellobiosidase (EC 3.2.1.91). Fungal cellulases have numerous industrial applications, for example in the brewing and fruit juice industries, where they are required for the removal of cellulose complexes, which have been found to interfere with the production process. These enzymes have also been utilized as digestive aids, particularly as supplements to animal feedstuffs. Cellulases are also widely employed in textile manufacturing, though problems can arise from the additional enzyme activities that may be present in industrial cellulase preparations. Commercially speaking, the main production organisms are strains of *Trichoderma reesei*.

Fungal xylanases are used extensively as digestive aids, where they are effective in combating problems associated with arabinoxylan components of plant-based feed materials. Essentially, the enzyme breaks down the β-1,4 bonds between xylose residues and reduces the intestinal viscosity of wheat-based diets. As with cellulolytic enzyme preparations, industrial xylanase preparations are typically multi-component preparations made up of a number of endo- and exo-acting xylanolytic enzymes. Xylanases are also widely used in paper manufacture, where they are used to degrade the lignin component in the pulp. In this instance there is a need for cellulase-free xylanolytic enzyme preparations.

Other fungal enzymes that are widely used include glucanases, lipases and hemicellulases, and the reader should refer to the reading list at the end of the chapter for further study material.

5.5.3 Fungal enzyme production strategies

Fungal enzyme production is mainly through the use of submerged fermentation strategies, although a second method known as solid-state fermentation or the Koji process is used extensively in Asia. We will concentrate on submerged fermentation strategies, of which a typical flow diagram is depicted in Figure 5.4.

Broadly speaking, the fermentation stages involved in the production of fungal enzymes are relatively similar. Large batch fermentations using inexpensive culture media are scaled up in a similar manner to antibiotic production. The downstream processing steps though can vary widely, and this is dependent on the ultimate process in which the enzyme preparation will be used. Similarly, those that are produced intracellularly, such as lactase, will require additional processing steps to those that are produced on an extracellular basis. Generally though, most industrial enzymes are produced extracellularly in large batch fermentations and require little in the way of downstream processing. In fact, fewer processing steps are preferable, as this will lead to a reduction in

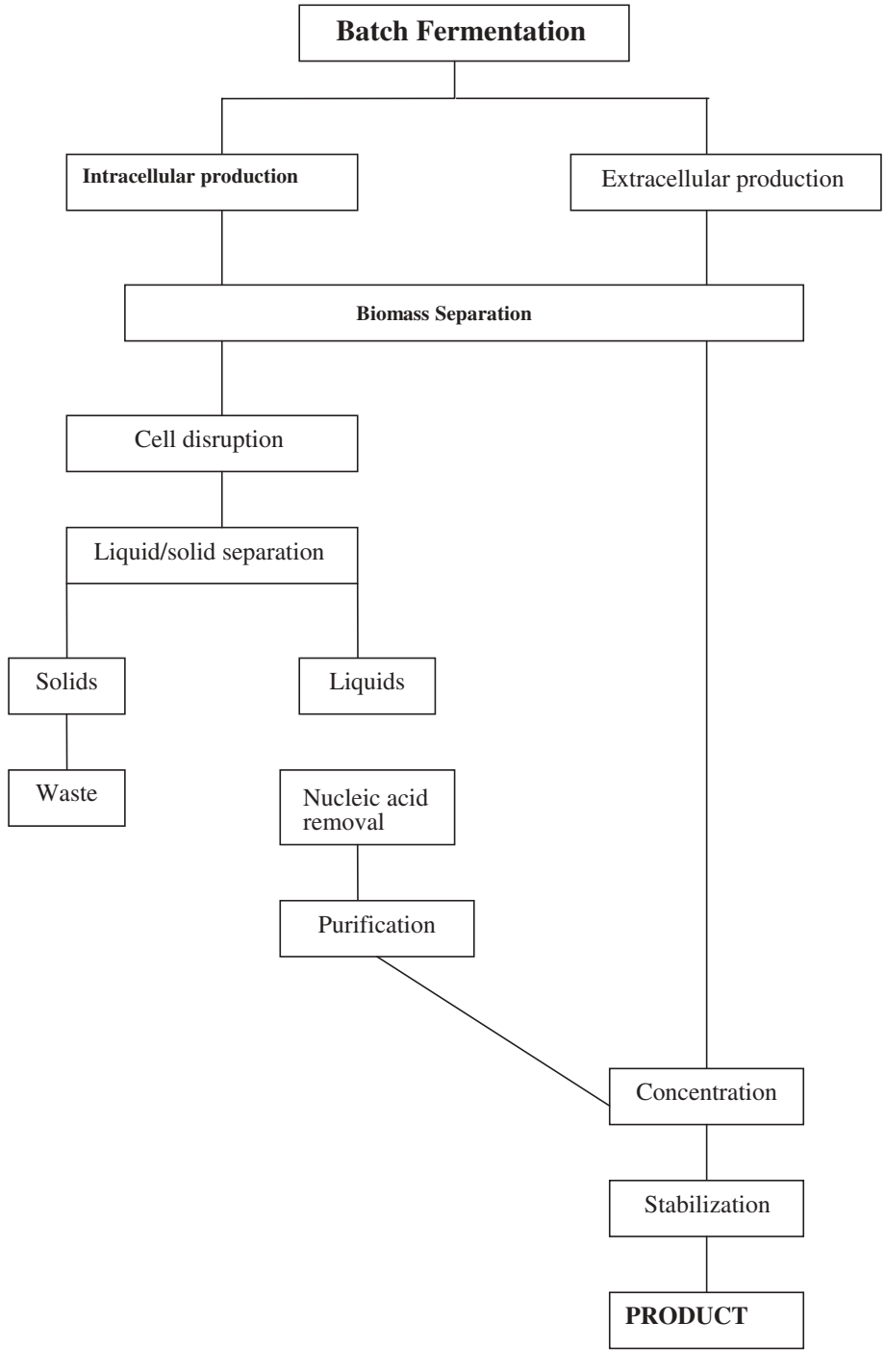


Figure 5.4 Flow diagram of fungal enzyme production

enzyme losses. Only enzymes and material likely to interfere with the catalytic process for which the enzyme is required will be removed. Unnecessary purification is to be avoided, as each step is costly in terms of equipment, manpower and enzyme loss. As a consequence, some industrial enzyme preparations are a simple mix of concentrated fermentation broth and additives to stabilize the preparation. If required though, a powder preparation can be prepared from a fermentation broth. Treatments such as salt or ethanol precipitation will result in the generation of a protein suspension from the spent culture media, which can then be filtered and dried to recover an extremely concentrated enzyme preparation.

5.6 Chemical commodities

Several industrially important chemicals are produced via biological processes using moulds and yeasts. In terms of world production volume, the most important of these is citric acid. From 1978 to 1984, the average rate of increase of total consumption of citric acid in Western countries was about 3.5 to 6 per cent per year and in 1995 the world market for citric acid was 500 000 tonnes.

5.6.1 Citric acid

Citric acid is the principal organic acid found in citrus fruit. To meet with increasing demands it is produced from carbohydrate feedstock by fermentation with the fungus *Aspergillus niger* and yeast of the genus *Candida*. The initial commercial production of citric acid was achieved using *Aspergillus niger* in a surface fermentation process. The development of the process of submerged fermentation in the 1950s was a major turning point in citric acid production. Citric acid's main use is as an acidulant in soft drinks and confectionery. A recent application of citric acid is as a metal complexing agent, to reduce oxidative metal deterioration and for metal cleaning. With the increasing requirement for citric acid, its production by fermentation is increasing continually, with about 500 000 tones of it being produced annually.

A number of fungi and yeasts have been used over the years for the production of citric acid but *Aspergillus niger* remains the preferred fermentation organism for commercial production. The main advantages for using this organism are its ease of handling, its ability to ferment a wide variety of cheap raw materials and its high yields.

A variety of raw materials such as molasses, starchy materials and hydrocarbons have been employed as substrate for the production of citric acid. Sucrose, cane molasses or purified glucose syrup from maize are sometimes used according to availability and price. Molasses has been acclaimed as a low-cost raw material and it contains 40–55 per cent of sugars in the form of sucrose, glucose

and fructose. There are considerable variations in the culture conditions reported in the literature for citric acid production by *A. niger*. To ensure high productivity it is essential that the culture medium contain major nutrients such as carbon, nitrogen and phosphorus and also trace elements. The fermentation process is also influenced by aeration temperature and pH.

The use of different carbon sources has been shown to have a marked effect on yields of citric acid by *A. niger*. *A. niger* can rapidly take up simple sugars such as glucose and fructose. Sucrose is usually the sugar of choice; at industrial scale the fungus possesses an extra cellular mycelial bound invertase, which under the acidic conditions of citric acid fermentation hydrolyses sucrose to its monomers. A sugar concentration of 14–22 per cent is considered the optimal level for maximum production yields. Lower sugar concentrations lead to lower yields of citric acid as well as the accumulation of oxalic acid.

The nitrogen sources for citric acid production by *A. niger* are generally ammonium sulphate, ammonium nitrate, sodium nitrate, potassium nitrate and urea. The presence of phosphorus in the fermentation medium has a profound effect on the production of citric acid. Too high a level of phosphorus promotes more growth and less acid production. Potassium dihydrogen phosphate (0.1 per cent) has been reported to be the most suitable phosphorus source. Maintenance of a low pH is essential for production; a pH below 2 is required for optimal fermentation. Citric acid fermentation is an aerobic process and increased aeration rates have resulted in enhanced yields and reduced fermentation times. Trace elements are also an important factor in the yields obtained in citric acid fermentation.

5.6.2 Industrial processes for the production of citric acid

A number of different fermentation processes exist for the production of citric acid, which we will outline.

The surface processes

Surface culturing was the first process employed for the large-scale production of microbial citric acid. Despite the fact that more sophisticated fermentation methods (submerged process) have been developed, surface culturing techniques are still employed as they are simple to establish and operate. Another advantage of this culturing method is that energy costs for surface fermentation are lower than those of submerged fermentation. The mycelium is grown as a surface mat in shallow 50–100l stainless steel or aluminium trays. The trays are stacked in stable racks in an almost aseptic fermentation chamber.

The carbohydrate sources (usually molasses) for the fermentation medium is diluted to 15 per cent sugars, the pH is adjusted to 5–7 and any required pre-

treatment is performed. After the addition of the nutrients, the medium is sterilized, cooled and pumped into the trays. Inoculation is performed by introducing spores, either by generating a spore suspension or by blowing spores over the surface of the trays along with air. Spores subsequently germinate and form a mycelial mat. The temperature is maintained at 28–30 °C and the relative humidity between 40 and 60 per cent.

During fermentation considerable heat is generated, necessitating high aeration rates. Air provides oxygen to the organism and also controls the fermentation temperature and the relative humidity. As the fermentation progresses, the pH decreases to below 2.0. If the fermentation pH rises to 3.0, oxalic acid and gluconic acid may be formed in considerable amounts. Fermentation progresses for 8–12 days, after which time the fermented liquid is poured out of the pans and separated from the mycelium for further processing.

Submerged fermentation

This process is now more popular for the commercial production of citric acid. It requires less space and is less labour intensive, and higher production rates are obtained. With submerged fermentation a stirred tank reactor or a tower fermentor may be used (Figure 5.5).

In view of the low pH level that develops during fermentation and the fact that citric acid is corrosive, the use of acid-resistant bioreactors is desirable. An important consideration with bioreactors designed for citric acid production is the provision of an aeration system, which can maintain a high dissolved oxygen level. With both types of bioreactor, sterile air is sparged from the base, although additional inputs are often used in tower fermentors.

The medium preparation in submerged fermentations involves appropriate dilution of the carbon source, pre-treatment addition of the appropriate nutrients and sterilization in line or in the bioreactor. Inoculation is performed by the addition of either a suspension of spores or precultivated mycelia. When spores are used they need to be dispersed in the medium, therefore addition of a surfactant is usually necessary. With pre-cultivated mycelia the inoculum size is usually about 10 per cent of the fresh medium. Air is bubbled (sparged) through the medium at a rate of 0.5–1.5 vvm throughout the fermentation. Under optimal conditions, fermentation is completed in 5–10 days. Submerged fermentation can be performed by continuous and fed batch modes, but generally it is carried out by the batch feed mode.

The Koji process or solid-state fermentation

The Koji process, developed in Japan, is the simplest process for production of citric acid. This process is the solid-state equivalent of surface fermentation. The

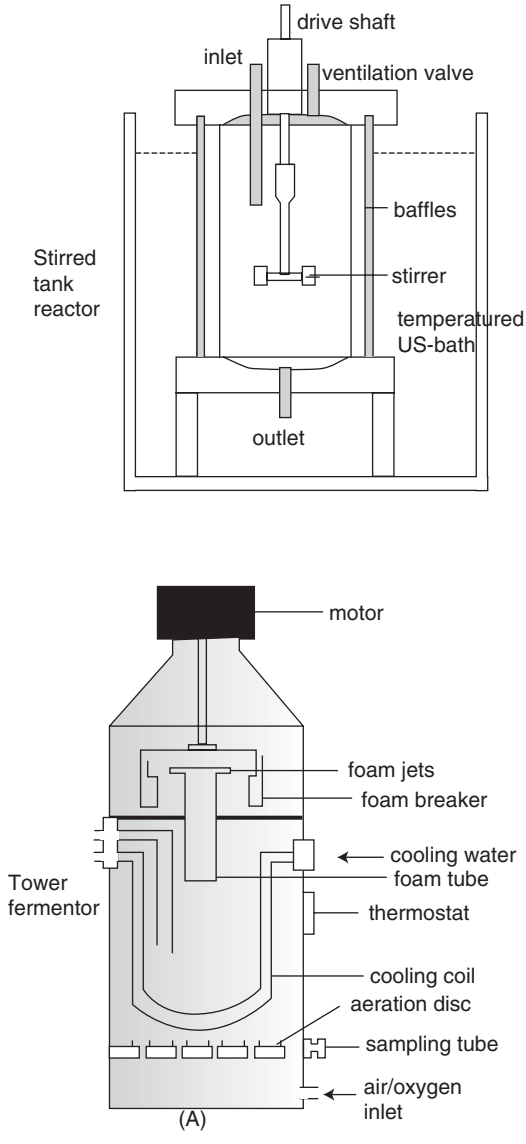


Figure 5.5 Types of reactor for citric acid production

raw materials used are sweet potato fibrous residues, rice or wheat bran and fruit wastes. The carbohydrate source is moistened with water to about 70 per cent moisture. The moist carbohydrate is then steamed for sterilization, placed in trays and inoculated using conidia of *A. niger*. The pH at the start of fermentation is 5.5. The starch is hydrolysed by amylase produced by the fungus and subsequently converted to citric acid. The fermentation is complete in 4–5 days. The main problem with this process is the presence of trace elements, which cannot be removed by standard methods.

Production of citric acid by yeast

Yeasts are also commercially employed in the commercial production of citric acid from various carbon sources. Yeast strains that are used in the production of citric acid include *Saccharomyces lipolytica*, *Candida tropicalis*, *Candida olephila*, *C. guillermondii*, *C. citroformans* and *Hansenula anomala*. There are a number of advantages when using yeast in comparison with filamentous fungi. Yeast can tolerate high initial sugar concentration; they are insensitive to trace metals and can thus ferment crude carbon sources without any treatment; they also have a great potential for being used in continuous culture and have a high fermentation rate. For commercial production of citric acid by yeast, tower fermentors with efficient cooling systems are employed. To inoculate the fermentation an inoculum is prepared in a smaller fermentor and is subsequently transferred into the production fermentor. The temperature of the fermentation is maintained between 25 and 37 °C, depending on the type of strain employed. The pH is generally more than 5.5, but can fall during fermentation. A continuous process for citric acid production using *Candida* cultured on cane molasses has been developed.

Citric acid metabolic pathways

The exact mechanism for citric acid production is not clearly understood, but involves an incomplete version of the tricarboxylic acid cycle. During the initial metabolism of glucose there may be an increase in cellular oxaloacetate levels, which decrease the catabolism of citrate by α-ketoglutarate dehydrogenase and simultaneously increase the rate of citrate synthetase. The condensing enzyme citrate synthetase brings about the biosynthesis of citric acid by the condensation of acetyl-CoA and oxaloacetic acid. This condensation of C2 and C4 compounds is the major route of citrate synthesis (Figure 5.6). Citrate synthetase has been shown to have allosteric regulation. Oxaloacetic acid for citric acid formation is achieved by way of the citric acid cycle and by an anaplerotic reaction for high yield of citric acid. Once the concentration of citric acid in the cells is high enough, the acid has to be excreted (see Figure 5.6).

Product recovery

Following the 5–10 days fermentation, the microbial cells are separated from the fermented liquor by centrifugation or filtration. Yeast-based fermentation liquors kept neutral with $CaCO_3$ or lime need to be acidified with mineral acid before this step. Citric acid is precipitated from the filtrate or supernatant as insoluble calcium citrate tetrahydrate by the addition of lime. The filtered washed calcium salt is treated with sulphuric acid in an acidulator. A precipitate of calcium sulphate is formed and filtered off. The remaining citric acid

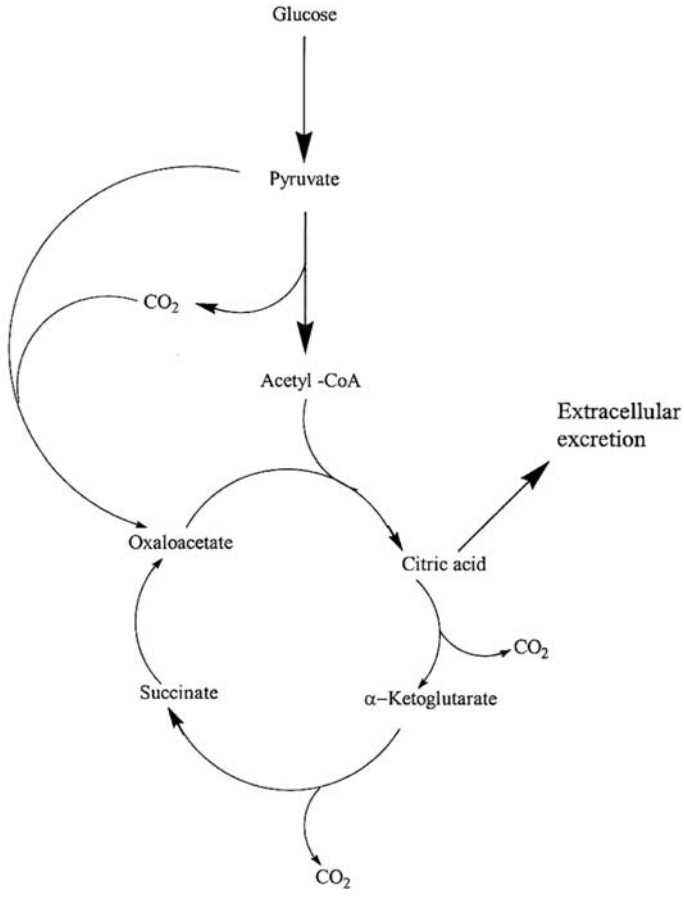


Figure 5.6 Citric acid biosynthetic pathway

solution is treated with active carbon, passed through cation exchangers and concentrated by evaporation before it crystallizes. Alternatively, citric acid may be extracted from the filtered broth using either tributyl phosphate or long chain secondary or tertiary amines. The acid is extracted into solvent at low temperatures and re-extracted into water at a higher temperature. The purified solution is concentrated and crystallized.

5.6.3 Itaconic acid production

Itaconic acid is used to alter the dyeing characteristics of vinyl polymers and also in the manufacture of polymers used in emulsion paints. Itaconic acid accumulation was originally observed in *Aspergillus itaconicus*; *Aspergillus terreus* and mutants of this strain are now more widely used. The main carbon sources

used in the commercial production of itaconic acid include glucose together with inorganic salts, purified molasses or media containing a portion of beet molasses. Calcium and zinc are also essential in the growth medium.

Fermentation process for production of itaconic acid

Once the medium is prepared and sterilized, inoculation is performed by the addition of either a suspension of spores or precultivated mycelia. Although surface fermentation in trays has been used, the submerged method is more widely preferred. Aerated and agitated stainless steel tanks are employed and provision for cooling is necessary. Fermentation temperatures for itaconic acid production are quite high, approximately 40 °C. The pH of the medium must be reduced to 2 to initiate production; once the accumulation of itaconic acid is well under way, higher yields are obtained if the medium is partially neutralized. The fermentation is highly aerobic and continuous aeration is required to minimize production losses. Following 72 hours of fermentation, yields of 60 per cent can be obtained based on carbohydrate source supplied.

Carbohydrate is metabolized, in *Aspergillus itaconicus* cells, by glycolysis to pyruvate, which is further converted through the citric acid cycle to aconitic acid. Aconitic acid is then converted to itaconic acid by the enzyme aconitic acid decarboxylase, an enzyme that has been reported to be extremely oxygen dependent.

Product recovery

The mycelium is separated from the fermentation medium by filtration and the resultant liquor clarified. The itaconic acid is then recovered by evaporation and crystallization, by ion exchange or solvent extraction.

5.6.4 Vitamin production

Vitamins are essential nutrients required in small quantities and have a documented and accepted value to the health of humans and animals. There is a large requirement for extra vitamins, other than those derived from plant and animal food sources, due to unbalanced food habits or processing, food shortage or disease. Added vitamins are either prepared chemically or biotechnologically via fermentation or bioconversion processes (see Table 5.4). Several vitamins are at the moment only produced via organic chemical synthesis. However, for many of these compounds microbiological processes for their production are rapidly emerging and some are now used commercially. Compounds such as riboflavin (B_2), ergosterol (provitamin D_2), cyanocobalamin (B_{12}), orotic acid

Table 5.4 Industrial production of vitamins

Vitamin	Chemical synthesis		Microbial synthesis			World Production (tons/year)
	Organic	Extraction	Bacterial	Fungal	Algal	
Vitamin B_1	+					2 000
Vitamin B_2	+		+	+		2 000
Niacin (B_3,PP)	+		+			8 500
Pantothenic acid (B_5)	+					4 000
Vitamin B_6	+					1 600
Biotin (B_8)	+		+			3
Folic acid (B_9)	+					300
Vitamin B_{12}			+			10
Vitamin B_{13}			+			100
Vitamin C	+		+			70 000
Vitamin A	+					2 500
Provitamin D_2				+		
Provitamin D_3	+	+				25
Vitamin E	+	+		+	+	6 800
Vitamin F		+		+	+	1 000
Vitamin K2	+					2

(B_{13}) the vitamin F group and vitamin C are now produced exclusively via fermentation.

Vitamin B_2 (riboflavin)

Riboflavin is commonly used in animal feed and human nutrition. It is produced by both synthetic and fermentation processes, with the latter recently increasing in application. The first fermentations were initially performed in 1965 but were terminated after three years as they proved more expensive than chemical processes. With improvement in producer organisms the microbial process was revived in 1975 and is now increasingly used for riboflavin production. Although bacteria (*Clostridium* sp.) and yeasts (*Candida* sp.) are also good producers, currently two closely related ascomycete fungi *Ashbya gossypii* and *Eremothecium ashbyi* are considered the best riboflavin producers. *Ashbya gossypii* is the preferred strain for production as *Eremothecium ashbyi* is genetically unstable. Soya bean oil and soya bean meal are the substrates most commonly used in *Ashbya gossypii* fermentations. Riboflavin production occurs during the late phase of growth, when all the glucose in the medium is

exhausted. While glucose remains in the medium, during the early phase of growth it is converted to lipid droplets, which are used later in riboflavin production. Supplementation of the culture medium with glycine or ribitol stimulates riboflavin formation. Both compounds are precursors in the riboflavin synthetic pathway and their effects suggest a limitation of central metabolites.

Vitamin D

Vitamins D_2 and D_3 are used as antirachitic treatments and large amounts of these vitamins are used in the fortification of food and feed. Vitamin D_2 (ergocalciferol) is obtained by the UV irradiation of yeast ergosterol (provitamin D_2) (Figure 5.7). Efficient fermentation processes for ergosterol accumulation have been established. *S. cerevisiae* accumulates high levels of sterols. Of about 20

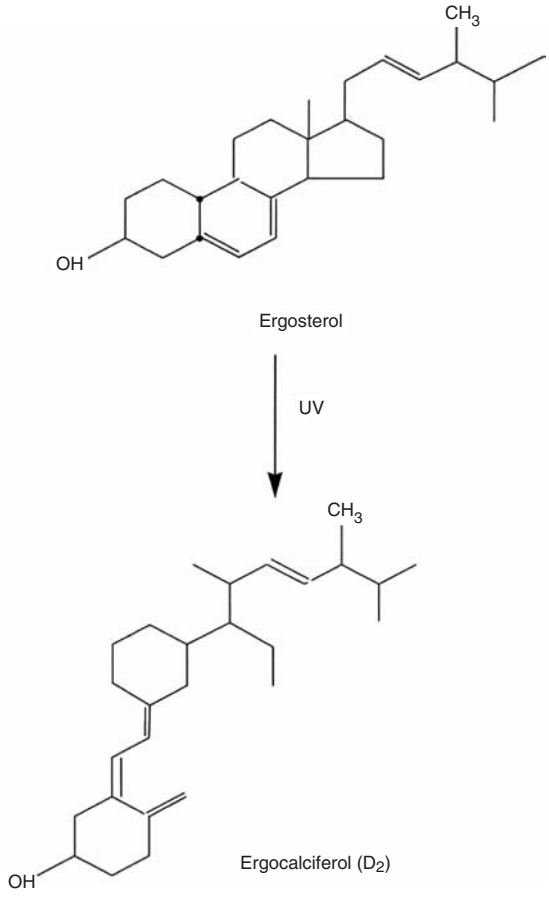


Figure 5.7 Vitamin D biosynthesis

sterols encountered in *S. cerevisiae*, ergosterol, ergosta-5, 7, 22, 24 (28)-tetraen-3β-ol, zymosterol and lanosterol are considered to be the major sterols, of which ergosterol makes up over 90 per cent.

Yeast cells consume carbohydrates as energy and carbon sources by aerobic and anaerobic metabolism. The concentration of carbohydrate and the supply of oxygen determine which metabolic pathway the yeast cells utilize. To overcome the repression caused by insufficient nutrients or an over-supply of carbohydrate, fed batch methods have been used in the process of ergosterol fermentation. In the yeast culture process glucose is the preferred carbon source, and when the glucose concentration reaches a low level the cell growth is confined. Then after a short period of adaptation cells continue to grow by consuming the ethanol, produced in the first phase, as the carbon source (termed biphasic growth). The whole process appears to be a two-phase process with the ergosterol content increasing when the specific growth rate is decreased.

5.7 Yeast extracts

Since ancient times both Western and Oriental cultures have used micro-organisms to transform or produce food. Fungi have a crucial role to play in the processing of many foods, improving the texture digestibility, nutritional value, flavour or appearance of the raw material used. The first industrial production of mico-organisms for nutritional purposes took place in Germany, when Torula yeast was produced and incorporated into soup and sausages. Yeast cells may be solubilized either partially or completely by autolysis and several other techniques. On further processing, the slurries can be converted into a variety of preparations and products, which are useful in the laboratory and as ingredients in food, feeds and fermentation media. Among the principal products are concentrates of yeast invertase and β-galactosidase, soluble yeast components in liquid, paste, powder or granular form, and isolated fractions of yeast cell constituents, such as protein and cell walls (glucan, manno-oligosaccharide, emulsifiers, mannoprotein), liberated by cells fractured mechanically. The major commercial products are clear water-soluble extracts known generally as yeast extract, autolysed yeast extract and yeast hydrolysate.

5.7.1 Yeast extract production

There are three distinct manufacturing practices for yeast extract production; autolysis, plasmolysis and hydrolysis. Autolysis is a process by which the cell components within the cells are solubilized by activation of enzymes that are inherently present in the cell. This is achieved by carefully controlling temperature, pH and time, with the careful addition of enzymes or reagents to stimulate degradation and release of the cell contents into the medium. The amount

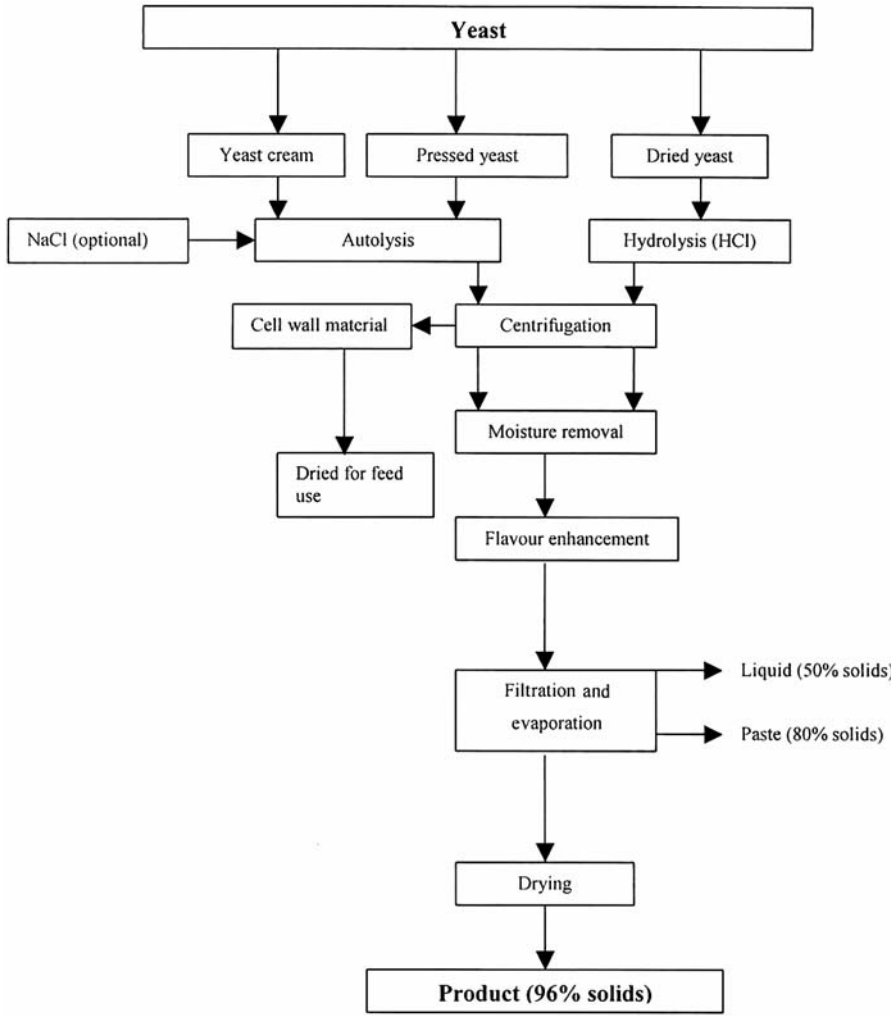


Figure 5.8 Yeast extract manufacturing process

of free amino acid present in the extract can serve as a rough guide to indicate the degree of hydrolysis. Free amino acids are known to, exert directly or indirectly, major influences on food flavour.

A yeast extract manufacturing process, outlined in Figure 5.8, which has gained more acceptance in Europe than the USA, is plasmolysis. In this process yeast cells are treated with salt and begin to lose water; the cytoplasm separates from the cell wall. When this happens, cells die and the degradative process begins. The advantages of this process are the fact that no specialized equipment is required and salt is relatively cheap to purchase and readily available. One drawback of this process is the high salt content of the extract. The

hydrolytic process utilizes the action of hydrochloric acid on yeast at specific temperatures and pressure. Hydrolysis is performed until the required concentration of free nitrogen is achieved; this usually takes 6–12 hours, and shorter more efficient hydrolysis can be achieved at higher temperature and pressure. The hydrolysate is neutralized with sodium hydroxide; the extract is then filtered and concentrated.

5.8 Enriched yeast

With increasing demand for organic and non-genetically modified additives in human and animal feeds, interest in the production of 'organic vitamins and minerals' has increased in recent years. One of the major concerns with the production of these organic products is economic viability. Thus to overcome this concern the incorporation into or enrichment of yeast (*Saccharomyces cerevisiae*) with minerals has been developed as a common industrial process. The two most commonly available of these yeast-type products are selenium yeast (Selplex®) and Chromium yeast (Cofactor III™).

5.8.1 Production of selenium yeast

Since selenium and sulphur exist in the same group within the periodic table and have many similar chemical characteristics, microbes such as *S. cerevisiae* have been shown to be unable to distinguish between the two atoms. Indeed, *S. cerevisiae* has the ability to metabolize selenium and incorporate it into molecules where sulphur would normally exist as the native atom. When propagated in a nutrient-enriched medium containing reduced levels of preformed amino acids, sulphur and selenium, as inorganic selenite or selenate salts, *S. cerevisiae* can utilize selenium as it does sulphur, resulting in the biosynthesis or various organic selenium compounds. The majority of the selenium in selenium yeast exists as analogues of the organo-sulphur compounds such as selenomethionine, selenocysteine and selenocystine. Small amounts are also thought to exist as selenohomocystenine, selenocystathione, methylselenocysteine, S-adenosyl-selenomethionine, the selenotrisulphide, selenogluthatione and various seleno-thiols, some of which are represented in Figure 5.9. The biosynthesis of such organo-selenium compounds is thought to be achieved through the biochemical pathways of organo-sulphur biosynthesis, which have been well characterized. These selenoamino acids can then be utilized by the protein synthesis machinery of the yeast cell and incorporated into protein molecules.

Crude protein, shown to account for approximately one-half of the dry weight of yeast, was characterized and was shown to be comprised of approximately 80 per cent amino acids, 12 per cent nucleic acids and 8 per cent ammonia. Manipulation of this data shows the total sulphur-containing amino acid

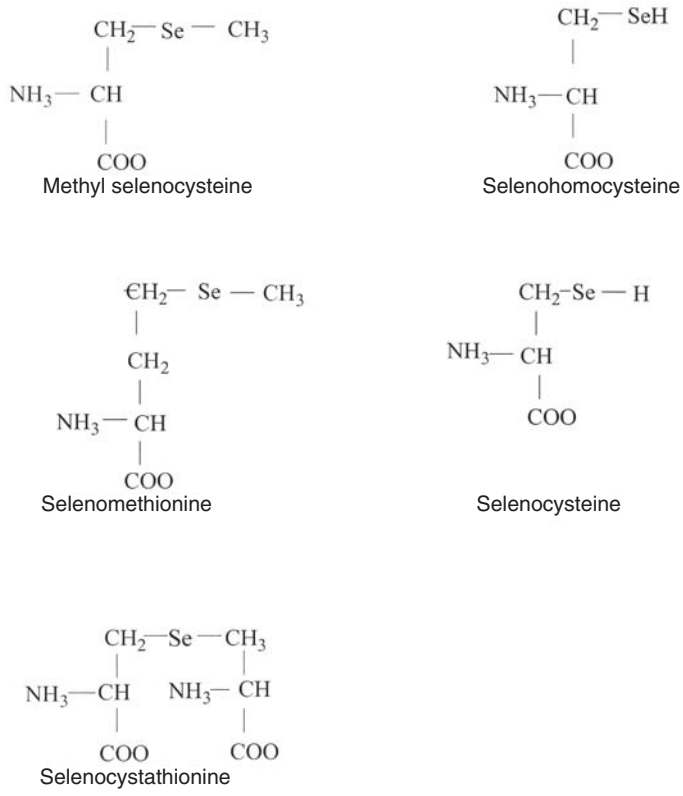


Figure 5.9 Selenium compounds in selenium yeast

content of *S. cerevisiae* to be 1.99 per cent (w/w) with 1.21 per cent as methionine and 0.78 per cent as cysteine. This represents 2600 ppm (parts per million) and 2080 ppm of organically bound sulphur in these forms. Studies have shown that up to 50 per cent of the methionine moieties in proteins can be replaced with selenomethionine while retaining biological activity. These findings would indicate that well in excess of 2000 ppm of yeast sulphur could be replaced by selenium, with possibly no adverse effects on protein synthesis or growth characteristics of the yeast cells. Therefore, if propagation conditions are carefully controlled, a growth pattern can be induced that allows the incorporation of selenium in the yeast to levels in excess of 2000 ppm, over 50 000 times the normal level of 0.04 ppm; the production process for selenium yeast is outlined in Figure 5.10.

Selenium yeast has many advantages over traditional inorganic selenium. These include a lessening of environmental concerns pertaining to the toxicity of selenium, as selenium yeast displays reduced toxicity over inorganic selenium forms. Another advantage associated with increased bioavailability is a reduc-

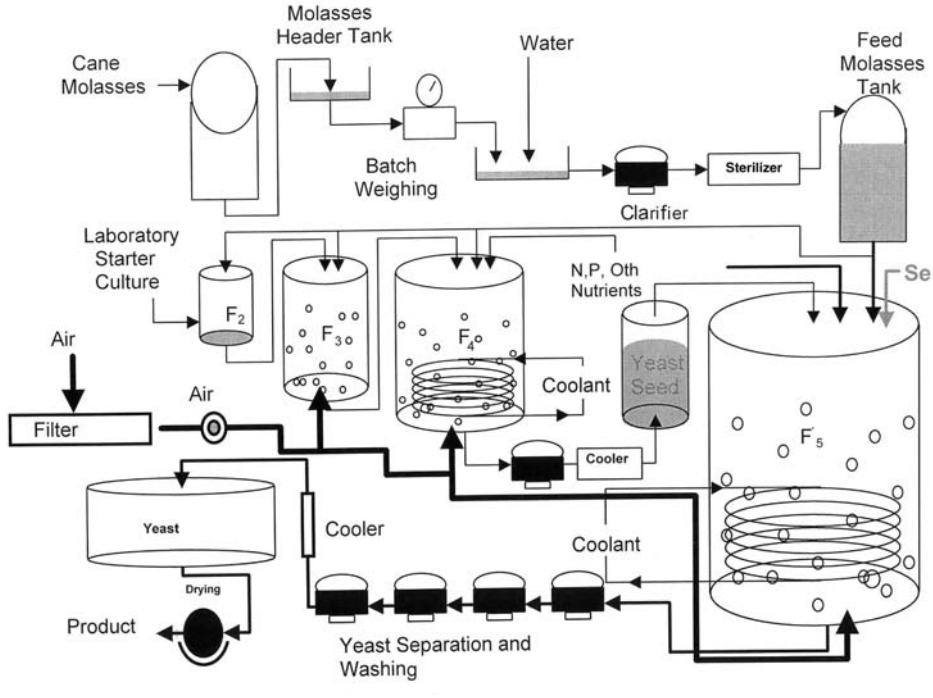


Figure 5.10 Selenium yeast production

tion in the level of unabsorbed selenium excreted by the faecal route. This would prevent toxic selenium build-up by concentration in the faeces and is of significance where intensive farming techniques are employed. Economically too, benefits are to be seen; with increased bioavailability, lower quantities of selenium would be required to supplement selenium-deficient diets, resulting in less expensive feeds.

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5.10 Revision questions

Q 5.1 Give three examples of both primary and secondary metabolites.

Q 5.2 Name the main cell wall component on which penicillins act.

Q 5.3 How does cyclosporin A function as an immunosuppresant?

Q 5.4 How do the so-called statins act to prevent cholesterol biosynthesis?

Q 5.5 Name five enzymes of economic importance and their applications.

Q 5.6 What fungus is the main producer of cephalosporins?

Q 5.7 Why is selenium an essential element?

6

The Biotechnological Exploitation of Heterologous Protein Production in Fungi

Brendan Curran and Virginia Bugeja

6.1 Fungal biotechnology

As befits their central role in the biosphere, fungi are key players in the profitable exploitation of biological systems by humans. Whether arising from the unconscious exploitation of fermentation in the making of wine, bread and beer by the ancients, the discovery and deliberate cultivation of edible fungi, the application of microbiology to the production of antibiotics, or the genetic engineering of recombinant proteins, fungi have been central players in the development of biotechnology. Many of these fascinating subjects are found elsewhere in this book. Here we restrict ourselves to a very specific brief: the production of biotechnologically relevant heterologous proteins in fungi.

Heterologous, or recombinant, proteins are produced when recombinant DNA technology is used to express a gene product in an organism in which it would not normally be made. Unlike recombinant DNA, which is made possible because the chemical and physical structure of DNA molecules are the same regardless of their origin, recombinant protein production is fraught with difficulty because of the complexities associated with the need to be overcome to transcribe and then translate the information in a heterologous DNA into an correctly folded protein with the appropriate biological activity.

Initially developed in the simple prokaryote *Escherichia coli*, heterologous protein expression technology now extends to scores of prokaryotic and eukary-

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otic host systems including Gram positive and negative bacteria, yeasts, filamentous fungi, insect cells, plants, mammalian cells and transgenic animals.

6.2 Heterologous protein expression in fungi

6.2.1 Overview

Heterologous gene expression systems are available for an extremely wide range of fungi including popular model organisms such as *Saccharomyces cerevisiae*, *Neurospora*, *Aspergillus* and *Penicillium*. Many have been developed for use in basic research; others for commercial exploitation. However, regardless of their intended use they all require (1) the insertion of the desired *heterologous DNA* coding sequence into appropriate regulatory sequences in specialized *expression vectors*, and (2) a *transformation* procedure for the introduction of the construct into the desired host species.

6.2.2 Heterologous DNA, vectors and transformation systems

Heterologous DNA

The DNA sequence due to be expressed into heterologous protein can be *genomic* in origin, thereby possessing introns and/or regulatory sequences from the original organism from which it has been cloned. Alternatively, it can be a *cDNA* sequence derived by reverse transcription of the heterologous mRNA, in which case it will lack introns and regulatory sequences. Filamentous fungi and many yeast species are capable of excising introns accurately from the mRNA transcripts of heterologous genes, and some filamentous species can even recognize heterologous regulatory signals from human DNA. However, efficient expression almost always requires the heterologous mRNA to be driven from the promoter of a strongly expressed host cell gene. Therefore, although heterologous genomic DNA has been successfully expressed in a small number of fungi, commercially important heterologous protein expression is normally initiated in these organisms by inserting the appropriate cDNA sequence into an expression vector that already encodes appropriate promoter and terminator sequences.

Vectors

In addition to the backbone of bacterial plasmid DNA, which is common to expression vectors in all systems (to facilitate DNA manipulation/large-scale plasmid purification in *E. coli*), fungal expression vectors carry

- a selectable marker for the intended fungal host
- a strong promoter to drive the production of the heterologous mRNA
- appropriate DNA sequences to ensure efficient termination of transcription and polyadenylation of the mRNA and
- appropriate sequences to ensure the correct initiation and termination of translation.

The most commonly used shuttle vectors for biotechnological applications in the yeast *S. cerevisiae* are autonomously replicating because they carry appropriate sequences (Figure 6.1) from yeast 2μ DNA – a native yeast plasmid. The vector is constructed in *E. coli* by inserting the cDNA of choice, in the correct orientation (see the arrow), into the multiple cloning site (MCS) between appropriate yeast promoter and terminator elements. The bacterial origin of replication (ori) and ampicillin resistance gene (AMP^R) allow for replication and selection in *E. coli*. The resulting plasmid, transformed into yeast, is selected by complementation of the appropriate auxotrophic marker (*His3*$^-$ in this example). The segment of yeast 2μ plasmid DNA ensures plasmid replication in the host cells. On the other hand, many of the vectors designed for use in methylotrophic yeasts such as *Pichia pastoris* and *Hansenula polymorpha* (see *methylotrophic yeast species* below), and in filamentous fungi, e.g. *Penicillium* and *Aspergillus* species, are integrative vectors (Figure 6.2(a)) that require the

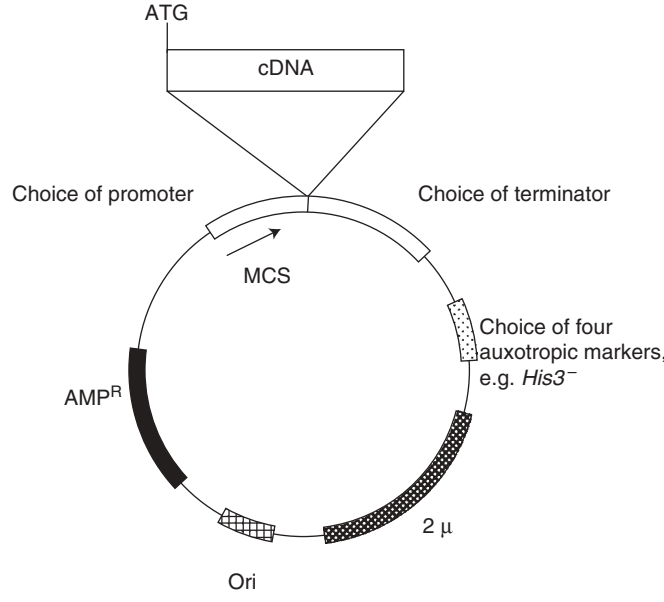


Figure 6.1 A generalized expression vector for use in *S. cerevisiae*

heterologous DNA to be incorporated into the host cell chromosomal DNA (Figure 6.2(b)). The vector (a) is constructed in *E. coli* by inserting the cDNA of choice, in the correct orientation, into the multiple cloning site (MCS) separating the promoter and terminator sequences of the host alcohol oxidase 1 gene (*AOX1*). (Note the presence of an extra 3′ segment of the AOX1 gene beyond the *His4* selectable marker.) The bacterial origin of replication (ori) and ampicillin resistance gene (AMP^R) allow for replication and selection in *E. coli*.

A linear fragment bounded by AOX1 sequences is then transformed into a *His4*$^-$ host. Integration by homologous recombination (b) results in the host AOX1 chromosomal locus being replaced by the *His4* selectable marker and the gene of interest driven by the AOX1 promoter.

Transformation and selection

The heterologous DNA molecules engineered in *E. coli* are transformed into the fungal host and colonies of transformants identified using appropriate selective agar plates. Although there are a number of dominant selectable markers conferring antibiotic resistance on the transformed cells (hygromycin B resistance is a particularly versatile one), many yeast and filamentous systems exploit auxotrophic marker complementation for selection. In these cases the plasmids carry the appropriate wild-type information to complement auxotrophic alleles (e.g. *Leu2*$^-$ *His3*$^-$ *Trp1*$^-$ etc.) in the host cells. The types of transformation process used include: enzymatically removing the cell walls and exposing the

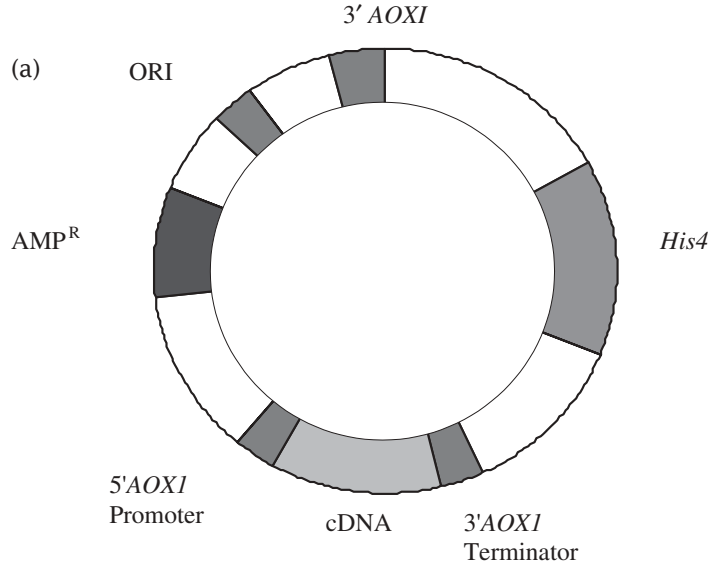


Figure 6.2 The structure (a) and integration (b) of a *P. pastoris* expression vector

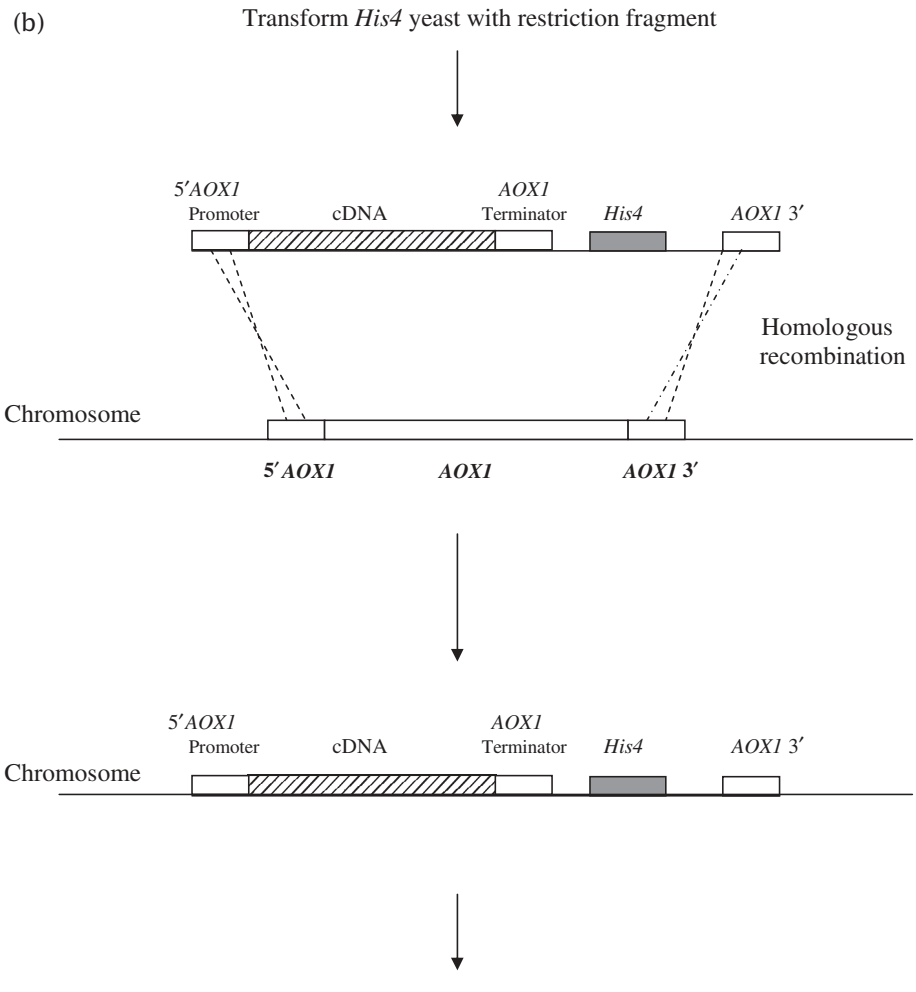


Figure 6.2 *Continued*

resulting sphaeroplasts to the DNA in the presence of calcium ions and polyethylene glycol; electroporation of yeast cells and fungal sphaeroplasts; and transformation of yeast cells by treating them with alkali cations (usually lithium) in a procedure analogous to *E. coli* transformation.

6.3 Budding stars

Despite the wide range of heterologous proteins expressed in fungi, protease contamination problems have bedevilled the development of high-level expression systems in filamentous fungi. Recent advances can now minimize the protease problem, but it is still yeasts, rather than their filamentous cousins, that

are the fungi responsible for the production of the majority of *heterologous* proteins with commercial relevance. Here we focus on the biotechnological development of the two most important members of this group: *Saccharomyces cerevisiae* and *Pichia pastoris*.

Saccharomyces cerevisiae

S. cerevisiae was the first eukaryotic cell engineered to express heterologous proteins because it shared with *E. coli* many of the characteristics that make the latter such a useful host for recombinant DNA technology. *Saccharomyces cerevisiae* grows rapidly by cell division, has its own autonomously replicating plasmid, can be transformed as intact cells and forms discrete colonies on simple defined media. In addition, *S. cerevisiae* can carry out post-translational modifications of expressed proteins – essential features of many heterologous proteins that *E. coli* is unable to provide. Furthermore, it secretes a small number of proteins into the growth medium, which, as we shall see, can be exploited to simplify the purification of heterologous proteins. Finally, it has a long safe history of use in commercial fermentation processes and, unlike *E. coli*, *S. cerevisiae* does not produce pyrogens or endotoxins. These parameters taken together make it particularly suitable for approval by regulatory bodies charged with the responsibility of ensuring the safe production of medically important heterologous proteins.

Despite the versatility of yeast expression systems, the production of high levels of biologically active heterologous proteins is still largely a matter of trial and error. The recovery of satisfactory levels of authentic heterologous protein depends on a number of factors including the type of expression vector used, the site of protein expression and the type of protein being expressed.

Expression vectors

There is a wide range of yeast cloning vectors available for use in *S. cerevisiae* but here we restrict ourselves to the self-replicating variety most commonly used for biotechnological applications (Figure 6.1). These YEp (yeast episomal plasmid) vectors are based on the ARS (autonomously replicating sequence) from the endogenous yeast 2μ (so called because of its unique length) plasmid. They are present at 20–200 copies per cell and under selective conditions are found in 60–95 per cent of the cell population. Integrative vectors similar to those used in *Pichia* (Figure 6.2(a) and (b)) can also be used in *S. cerevisiae*, and indeed one variant of this uses homologous recombination to target multiple copies of the gene construct into middle-repetitive DNA sequences generated by the activity of the Ty transposable element. However, in the vast majority of cases plasmid-borne expression vectors are used commercially.

Regulating the level of heterologous mRNA in host cells

The overall level of heterologous mRNA in the cell is determined by the copy number of the expression vector, the strength of the promoter used to drive transcription and the stability of the specific mRNA sequence.

Expression vectors based on YEp technology have a high copy number but require selective conditions to ensure their stable inheritance. High-level mRNA production is also dependent upon the type of promoter chosen to drive expression (Table 6.1). The most frequently encountered are based on promoters from the highly expressed genes that encode glycolytic enzymes. These include phosphoglycerate kinase (*PGK*), alcohol dehydrogenase 1 (*ADH1*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), all of which facilitate high-level constitutive mRNA production. However, high-level constitutive expression can be disadvantageous when the foreign protein has a toxic effect on the cells. This problem can be circumvented in most cases by using a regulatable promoter to induce heterologous gene expression after cells have grown to maximum biomass. There are a number of regulatable promoters available. One of the most useful ones is based on the promoter of the galactokinase gene (*GAL1*), which undergoes a 1000-fold induction when glucose is replaced by galactose in the medium.

Regardless of the choice of promoter, it is important that transcription of the heterologous mRNA is terminated properly, otherwise abnormally long mRNA

Table 6.1 Promoters used to direct heterologous gene expression in *S. cerevisiae*

Promoter	Strength*	Regulation	Example of heterologous gene expressed using promoter
PGK, 3-phosphoglycerate kinase	++++	Constitutive	Human β-interferon
ADH1, alcohol dehydrogenase 1	+++	Constitutive	Human β-interferon
GAPDH, glyceraldehyde-3-phosphate dehydrogenase	++++	Constitutive	Human epidermal growth factor
GAL1, galactokinase	+++	1000× induction	Calf chymosin by galactose
PHO5, alkaline phosphatase	++	500× repression	Hepatitis B surface antigen by phosphate
CUP1, copperthionein	+	20× induction by copper	Mouse IG kappa chain

* Relative levels of mRNA expression when the promoter is active.

molecules, which are often unstable, can be generated by read-through along the plasmid DNA. It is for this reason that expression vectors frequently contain the 3′ terminator region from a yeast gene (e.g. *CYC1*, *PGK* or *ADH1*) to ensure efficient mRNA termination (Figure 6.1).

Ensuring high-level protein production

As is the case with mRNA molecules, the level of heterologous protein produced by the host depends upon the efficiency with which the mRNA is translated, and the stability of the protein after it has been produced.

It is vitally important to address control of protein translation and subsequent translocation when choosing the expression vector. The sophisticated translation initiation mechanism found in mammalian cells is absent from yeast. It is therefore necessary to engineer away regions of base pair repeats (dyad symmetry) and non-coding AUG triplets in the leader sequence of heterologous mRNAs upstream of the AUG encoding the first methionine in the protein in order to ensure efficient initiation of translation. Once expressed, some proteins form insoluble complexes in *S. cerevisiae* but many others do not. Other proteins can be produced as denatured, intracellular complexes, which can be disaggregated, and renatured after harvesting. The first recombinant DNA product to reach the market was a hepatitis B vaccine produced in this way. Some proteins are rapidly turned over by the ubiquitin degradative pathway in the cell; others are degraded by vacuolar proteases. This can be especially true during cell breakage and subsequent purification. The powerful tools provided by a detailed knowledge of yeast genetics and biochemistry can be used to minimize this problem in *S. cerevisiae*.

The use of protease-deficient host strains can improve both the yield and the quality of heterologous proteins. One mutant (*PEP4-3*) is widely used because it is responsible for the activation of inactive vacuolar zymogen proteases; in its absence a wide range of proteinase activities is therefore prevented. Even more impressively, our detailed knowledge of the yeast secretory pathway can be exploited to genetically engineer the heterologous protein so that it is smuggled out of the cell before it can be degraded by either vacuolar proteases or the ubiquitin degradative pathway. Indeed, secretion not only minimizes the exposure of heterologous proteins to protease activity but, because *S. cerevisiae* only secretes a handful of proteins, it also facilitates the recovery and purification of heterologous proteins. Two of these (invertase and acid phosphatase) are targeted to the periplasmic space, which lies between the cell membrane and cell wall; the other two (α factor and killer toxin) are secreted out beyond the wall into the culture medium (Table 6.2). Entry into the secretory pathway is determined by the presence of short hydrophobic ‘signal’ sequences on the N-terminal end of secreted proteins. The DNA sequence for these signal peptides

Table 6.2 Signal sequences used to direct secretion of heterologous proteins from *S. cerevisiae*

Signal sequence	Cellular location of gene product	Example of secreted heterologous protein
Invertase	Periplasm	α-1-antitrypsin
Acid phosphatase	Periplasm	β-interferon
α-factor mating pheromone factor	Culture medium	Epidermal growth
Killer toxin	Culture medium	Cellulase

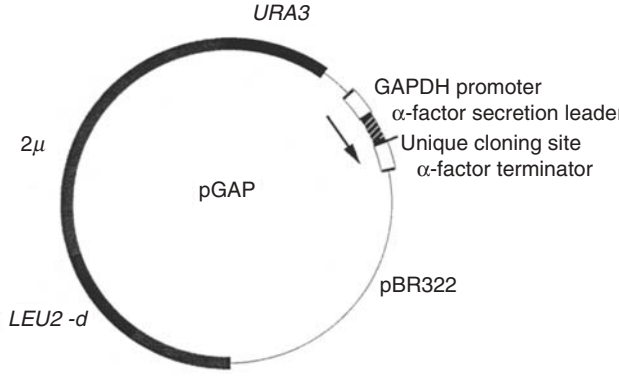


Figure 6.3 A secretion vector for use in *S. cerevisiae* (J. Travis, M. Owen, P. George, R. Carrell, S. Rosenberg, R. A. Hallewell and P. J. Barr, *J. Biol. Chem.*, 1985, **260**: 4384)

can be genetically engineered onto the DNA sequence for the heterologous protein of choice – thereby ensuring that it is targeted for export after being synthesized. The 'signal' sequences from all four of the *S. cerevisiae* secretion proteins have been used in this way with varying degrees of success. A secretion vector that encodes the α-factor signal peptide is shown in Figure 6.3. This vector contains *LEU2-d* and *URA3* yeast selectable marker genes, pBR322 sequences for amplification in *E. coli* and 2μ sequences for autonomous replication in yeast. The expression 'cassette' contains a unique cloning site flanked by GAPDH promoter, α-factor secretion leader and α-factor terminator sequences. Transcription initiation is indicated by the arrow. A number of medically important proteins, including insulin, interferon and interleukin 2, have been successfully secreted using this type of signal peptide.

Ensuring authentic protein structure and function

The objective of heterologous gene expression for commercial purposes is not just the high level transcription and translation of the appropriate DNA sequence. Proteins are produced because of their structure and therefore what is required is appropriately folded, biologically active, authentic protein molecules. Many proteins of therapeutic importance undergo sophisticated post-translational modifications in mammalian cells. These vary from the removal and/or addition of small chemical moieties such as the removal of the N-terminal methionine, or the addition of an acetyl group to the N-terminal amino acid (acetylation), through the addition of large lipid molecules to generate lipo-proteins, to the complex addition of countless sugar moieties to proteins as they are synthesized and passed through the cell's endoplasmic reticulum (ER) and golgi apparatus to produce glycosylated proteins. Simple prokaryotic expression systems such as *E. coli* are unable to carry out many of these processes and it was for that reason that eukaryotic expression systems were developed.

Although a simple eukaryote and therefore not ideal in all respects, *S. cerevisiae* offers solutions to at least some of these problems. When *E. coli* failed to produce properly acetylated human superoxide dismutase, *S. cerevisiae* obliged by intracellularly expressing a soluble active protein identical to that found in human tissue – complete with an acetylated N-terminal alanine. As for ensuring that proteins are folded correctly, that the N-terminal methionine removed, and added, sugar residues, the secretion system offers possible solutions: a direct comparison between the intracellular production and extracellular secretion of prochymosin and human serum albumin resulted in the recovery of small quantities of mostly insoluble, inactive protein when they were produced intracellularly but the recovery of soluble, correctly folded, fully active protein when they were secreted.

Secretion can also be used to produce proteins that have an amino acid other than methionine at their N-terminus. If a secretory signal is spliced onto the heterologous gene at the appropriate amino acid (normally the penultimate one) then the N-terminal methionine, which is obligatory for translation initiation, will be on the *secretory signal*. Proteolytic cleavage of this signal from the heterologous protein in the endoplasmic reticulum (ER) will generate an authentic N-terminal amino acid (Figure 6.4). Glycosylation in yeast is of both the N-linked (via an asparagine amide) and O-linked (via a serine or threonine hydroxyl) types, occurring at the sequences Asn–X–Ser/Thr and Thr/Ser respectively. However, it is important to note that the number and type of outer core carbohydrates attached to glycosylated proteins in yeast are different to those found on mammalian proteins. Therefore if the protein is being produced for therapeutic purposes they may cause unacceptable immunogenicity problems. One approach to overcoming this problem is to prevent glycosylation of the protein by using site-directed mutagenesis of the DNA in *E. coli* to alter one

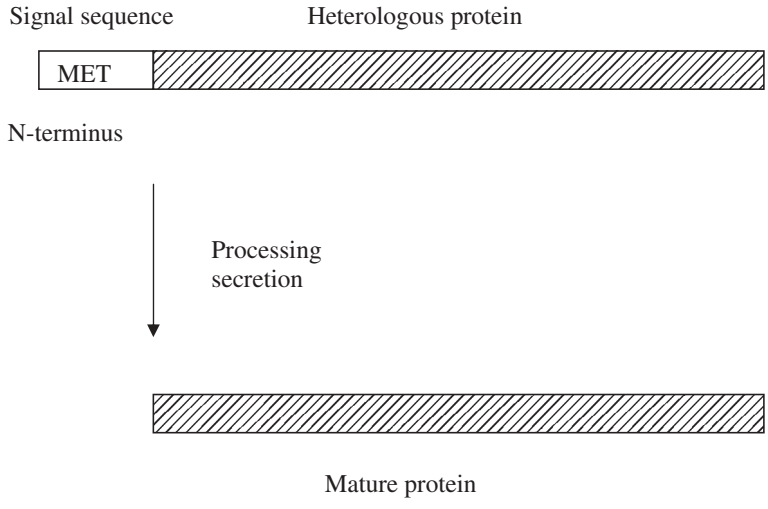


Figure 6.4 Cleavage of a secretory signal sequence from a heterologous protein

amino acid, thereby removing the glycosylation recognition site before expressing the protein in *S. cerevisiae*. This strategy was successfully used to produce urokinase type plasminogen activator.

Limitations

Despite its ability to express a wide variety of proteins, *S. cerevisiae* has limitations. Its very primitive glycosylation system frequently hyperglycosylates heterologous proteins. Moreover, the production of alcohol during glucose metabolism limits the generation of biomass and therefore heterologous protein product. However, other yeast genera have a number of advantages over *S. cerevisiae* in these respects and have therefore been developed as alternative host systems for the production of heterologous proteins (Table 6.3).

6.4 Methylotrophic yeast species

Collectively referred to as methylotrophs, a small number of yeast species belonging to the genera *Candida*. *Torulopsis*, *Pichia* and *Hansenula* share a specific biochemical pathway that allows them to utilize methanol as a sole carbon source. The promoters of the genes that encode the enzymes for this pathway are extremely strong and exquisitely sensitive to the presence or absence of methanol, making them ideal for the regulation of heterologous gene expres-

Table 6.3 A comparative overview of *S. cerevisiae*, *P. pastoris* and *H. polymorpha* as hosts for heterologous protein production

	S. cerevisiae	*P. pastoris*	*H. polymorpha*
Cell density	+	+ + +	+ + +
Glycosylation	Hyperglycosylation	No hyperglycoslyation	No hyperglycoslyation
Optimum growth temperature	25 °C	32 °C	43 °C
Most frequently used vector system	High-copy-number replicative	Low-copy-number integrative	High-copy-number integrative
Secretion system	Yes	Yes	Yes

sion. Offering the ease of genetic manipulation associated with *S. cerevisiae*, these species have a number of advantages over their ethanol-producing cousin. They grow to much higher cell densities in fermenters due to the absence of toxic levels of ethanol; they use integrative vectors, which removes the need for selective media to be used in fermenters whilst at the same time offering greater mitotic stability of recombinant strains. Most importantly, they have a more authentic type of glycosylation pattern for heterologous products. Of these species *P. pastoris* and *H. polymorpha* have a well-established track record in fermentation technology and biotechnologically important proteins produced by these yeasts have either already entered the market or are expected to do so in the near future.

6.4.1 *Pichia pastoris*

With a popular commercial kit (Invitrogen, San Diego*)* widely available, *P. pastoris* has been used to express over 100 heterologous proteins for both research and commercial purposes. Selection of transformants for heterologous gene expression commonly relies on complementation of an auxotrophic *his4* marker in the host cells, although a number of dominant selectable markers are also currently available. Unlike *S. cerevisiae*, integrative vectors (as opposed to autonomously replicating plasmids) are normally used in this yeast species. The gene of interest is spliced in between the promoter and terminator sequences of the *AOX1* gene in an *E. coli* vector, which also carries the $His4^{+}$ gene and further downstream of this the 3′ end of the *AOX1* gene (Figure 6.2). A linear fragment bounded by AOX1 sequences is then transformed into a $His4^{-}$ host. This DNA construct can then undergo homologous recombination targeting the gene of interest into the chromosomal locus of the *AOX1* gene (Figure 6.2(b)). Such cells can grow either on methanol using an alternative alcohol oxidase locus, in

which case the heterologous protein is continuously expressed, or on glucose, in which case the heterologous gene is repressed until induced by methanol. The tight level of regulation allows for extremely precise control of the expression of the heterologous gene. Integrative vectors are also available that target the constructs to the *His4* locus. Quite apart from the fact that this easily regulated promoter has practical advantages over the more cumbersome galactose-inducible ones used to regulate heterologous expression in *S. cerevisiae*, *P. pastoris* is also regarded as a more efficient and more faithful glycosylator of secreted proteins. The most widely used secretion signal sequences include the *S. cerevisiae* α-factor prepro sequence and the signal sequence from *Pichia's* own acid phosphatase gene.

Many proteins of therapeutic importance have been successfully made using both intracellular and extracellular production in *Pichia*. These include the following.

- Proteins involved in the prevention and treatment of clots – one of the biggest causes of illness in the Western world. For example, a production strain, which secretes 1.5 g/l of the anticoagulant hirudin varient 2 (HV-2) into the culture medium, has been developed using the *S. cerevisiae* α-factor prepro signal sequence.
- Peptide hormones and cytokines. A number of these have been expressed including a new designer cytokine expressed in *Pichia*, which consists of interleukin-6 and a soluble form of its receptor. This has been shown to be capable of expanding haematopoietic progenitor cells, thereby offering new approaches to treating blood cell deficiencies.
- Protein vaccines, including a hepatitis B vaccine, which is already on the market.

6.4.2 *Hansenula polymorpha*

Similar in many respects to *Pichia*, this methylotrophic yeast offers a number of differences, which make it particularly attractive for industrial applications. These include the following.

1 *It is more thermotolerant. H. polymorpha* can quite happily tolerate growth temperatures up to and including 43 °C, which is far higher than the 30 °C maximum of *P. pastoris*. Not only does this allow for growth to persist at higher fermenter temperatures, it greatly accelerates a high-yield fermentation process.

2 *It facilitates multicopy integration of expression vectors.* Although, like *Pichia*, *Hansenula* transformation systems are based mainly on integrative vectors that carry selectable auxotrophic markers, the vectors undergo non-

homologous recombination, thereby ensuring that multiple copies of the heterologous gene are integrated into the host genome. Not only does this facilitate very high levels of heterologous gene expression, but in multiple auxotrophic host strains it offers the possibility of integrating several different genes, allowing co-expression of two or more recombinant proteins. Cells containing optimal stoichiometric amounts can then be selected. Functional haemoglobin A consisting of two α and two β chains was produced in this way. This technology can also be directed towards the generation of recombinant *H. polymorpha* strains capable of undertaking biotransformation reactions. One such biocatalytic recombinant containing 30 copies of a spinach-derived glycolate oxidase (*GO*) gene and 15 or 25 copies of the catalase T gene (*CTT1*) from *S. cerevisiae* efficiently converted exogenously added glycolate to glyoxylate.

3 *It eliminates the need for methanol.* The most commonly used *H. polymorpha* gene promoter is derived from the methanol oxidase gene (*MOX1*). However, unlike the *AOX1* promoter in *Pichia*, the *MOX1* promoter becomes derepressed as glucose becomes exhausted. Although this removes the precise regulation offered by *Pichia* (because expression can start before methanol is added to the cells), it presents the opportunity of replacing methanol with glycerol. In the absence of glucose this carbon source is sufficient in order to achieve high level expression. This derepression mechanism, which is not found in *Pichia*, also circumvents the complications arising when an expression cassette integrates into the *MOX1* locus in *H. polymorpha*. Unlike *Pichia*, where the target for integration is just one of two homologous *AOX* genes, integration into the *unique MOX1* locus removes the option of using methanol as a sole carbon source. However, the derepression mechanism means that constitutive production is still possible in such transformants by simply using glycerol as the sole carbon source.

6.5 Case study – hepatitis B vaccine – a billion dollar heterologous protein from yeast

With sales figures in excess of two billion dollars per annum, recombinant hepatitis B sub-unit vaccines produced in yeast are one of the major success stories of molecular biotechnology. Produced as a heterologous protein originally in the yeast *S. cerevisiae* and more recently in both *P. pastoris* and *H. polymorph*, the phenomenal success story of the hepatitis B vaccine makes it an ideal candidate with which to illustrate the research and development of a biotechnologically important heterologous protein.

6.5.1 Hepatitis: a killer disease and a huge market opportunity

Hepatitis B, a double-shelled virus in the class Hepadnaviridae (Figure 6.5), is responsible for the death of more than 250 000 people worldwide every year. The liver infections caused by this organism can manifest in one of two different modes: either acute or chronic viral hepatitis. In acute hepatitis the virus is completely cleared from the body when the symptoms disappear. In chronic hepatitis, the virus persists in the liver after infection, and the patient becomes a carrier of the disease. The chronic mode of the infection is regularly associated with progressive cirrhosis and primary hepatocellular carcinoma. Worldwide a staggering 200 million men, women and children carry the disease.

In the vast majority of cases antiviral interferon therapy is either unavailable or, in the cases where symptoms have already developed, ineffective. Therefore vaccination offers the only useful medical intervention with respect to this disease. In brutal biotechnological terms the production of an effective hepatitis B vaccine constitutes a huge market opportunity. This is why so many companies develop and sell these products.

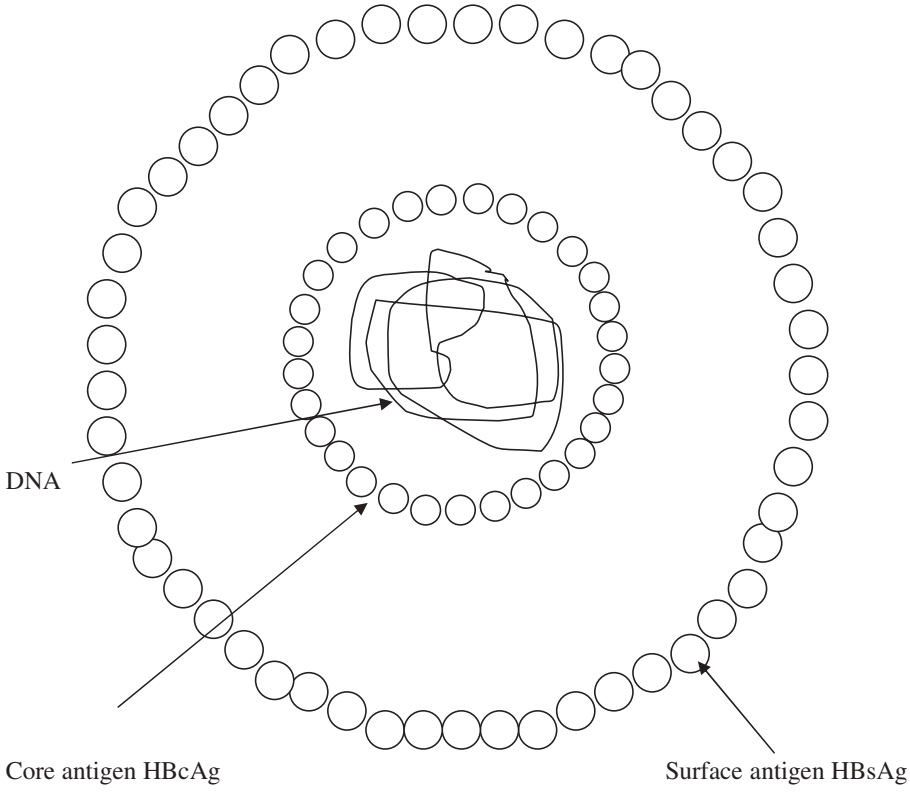


Figure 6.5 The hepatitis B virus

6.5.2 A vaccine from infected carriers of the disease

The first commercially available HB vaccine was prepared from protein particles of viral origin isolated from the plasma of chronic carriers of the disease. These extremely immunogenic 22 nm sub-viral particles (non-infective by-products of viral replication) consist of multiple molecules of a glycoprotein, called the hepatitis B surface antigen (HBsAg), embedded in a phospholipid membrane of cellular origin. These non-infective particles appear as if they are complete 42 nm viral particles to the immune system. They therefore elicit a strong immune response against the coat of the virus and confer resistance to subsequent viral infection.

First licensed in 1981 and used for over 10 years in the USA, this vaccine was safe, effective and well tolerated. It was less than ideal, however, because it depended upon a continuous supply of plasma, and required extensive processing and safety testing. Although expensive to produce the vaccine would have been more widely acceptable except for unbiased fears that, despite elaborate safety precautions to prevent contamination, it had the potential to contain infective hepatitis B viruses, or other blood borne diseases (such as HIV), originating from the donor plasma.

Biotechnologists therefore turned to recombinant DNA technology to circumvent the problems associated with the human-derived product. However, given that genetic manipulation was in its infancy in the early 1980s but that heroic efforts were needed to express even a simple human protein in *E. coli*, the expression of an effective heterologous hepatitis B vaccine was a tall order indeed. Such a challenging objective required genetic engineers to separate the DNA sequence encoding the HBsAg protein from the rest of the viral genome and then to arrange to have the information transcribed and translated into an *immunogenic protein* in a suitable host cell.

6.5.3 Genetically engineering a recombinant vaccine

Using the previously sequenced 3200-base hepatitis B genome, the short DNA sequence encoding the 226 amino acids that comprise the major surface protein was isolated, spliced in frame with a strong promoter in an *E. coli* expression vector and transformed into the appropriate cells. Although the viral protein was expressed, it was not glycosylated. Furthermore, the host cells failed to produce the 22 nm phospholipid–protein particles. Given that the unassembled human HBsAg protein was known to be 1000 times less immunogenic than the 22 nm plasma-derived particles scientists were not surprised when the *E. coli* recombinant protein failed to elicit an appropriate immune response in animals.

However, undaunted by this failure, scientists then attempted to exploit recombinant DNA technology that had just been developed to facilitate basic gene cloning in the simple eukaryotic organism *S. cerevisiae*. It was hoped that

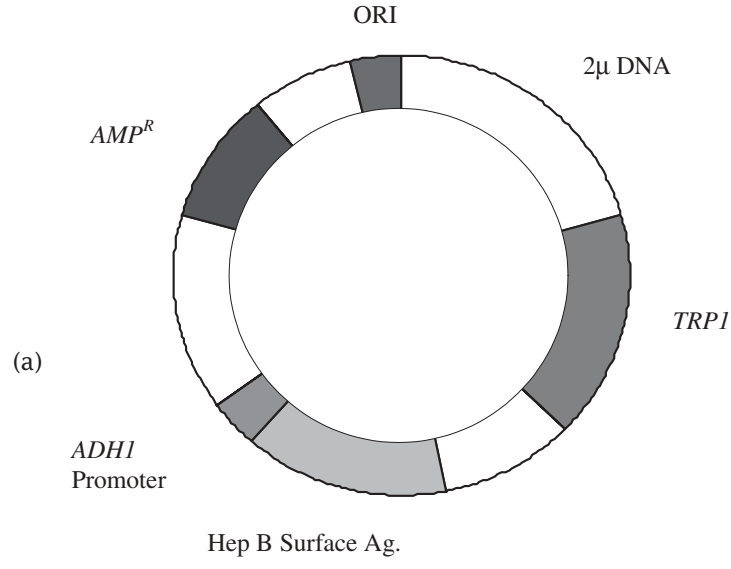
this yeast, a eukaryote capable of glycosylating and secreting proteins, would be able to produce immunogenic particles of glycosylated proteins where *E. coli*, a prokaryote, had failed. In a proof of principle experiment the DNA encoding the HBsAg was spliced downstream of the yeast alcohol dehydrogenase (*ADH1*) promoter in an *E. coli*-based shuttle vector carrying a 2μ replication origin and the *TRP1* gene (Figure 6.6(a)). After transformation and selection in a *trp1*$^{-}$ host not only were the resulting transformants found to express substantial levels of HBsAg protein, but the proteins aggregated into phospholipid particles similar to those found in the plasma from human carriers of the disease. Unlike the majority of HBsAg proteins synthesized in humans, however, the yeast-expressed protein lacked glycosylation, and rather than being secreted it accumulated inside the cells. Despite the lack of appropriate glycosylation, these particles elicited the appropriate immunological response when tested in animals, indicating that glycosylation was not needed for assembly of the particles or for immunogenicity.

6.5.4 From proof of principle to industrial scale-up

Having used a basic expression vector to demonstrate that yeast could produce immunogenic 22 nm phospholipid–protein particles, thereby circumventing the problems associated with the blood-borne source of the vaccine, an improved expression vector (Figure 6.6(b)) was developed as a prelude to industrial scale-up of vaccine production. A comparative analysis of the industrial vector and the vector used in the proof of principle experiments illustrates many of the molecular subtleties associated with ensuring high-level heterologous protein production in yeast.

As can be seen in Figure 6.6, both plasmids are shuttle vectors carrying segments of plasmid DNA from *E. coli* and both are based on the yeast 2μ plasmid. However, whereas the proof of principle vector has a *TRP1* marker, the industrial one has a *LEU-2d* gene. This gene has a truncated promoter and, as it is ineffectively transcribed, the cell requires a higher copy number of the plasmid encoding it in order to be able to grow in the absence of leucine. On average each cell has 150–300 copies of a *LEU-2d*-carrying plasmid per cell, as against approximately 30 copies of the *TRP1*-carrying plasmid. The industrial vector also has a terminator sequence to ensure efficient termination, whereas the proof of principle vector lacks such a sequence. Finally, the reasonably strong *ADH1* promoter of the proof of principle vector has been replaced in the industrial one by an extensively characterized, highly efficient *GPDH* promoter.

In short, in addition to the basic expression modules found in the proof of principle vector, the industrial one has DNA sequences to ensure a higher plasmid copy number, more efficient transcription initiation and more efficient termination of transcription. All of these lead to high-level mRNA production, thereby ensuring that the level of protein expressed is significantly higher in the industrial strain than in the proof of principle host.



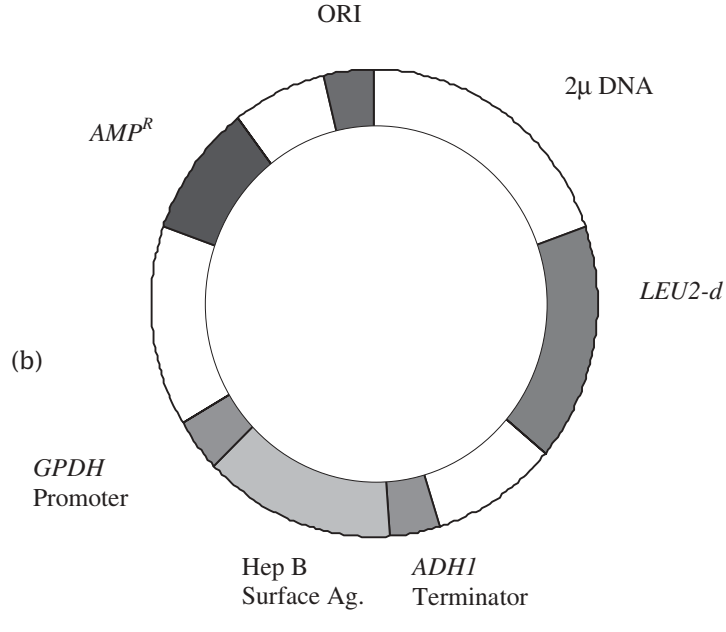


Figure 6.6 Proof of principle and scale-up plasmids

6.5.5 Much more than a development in expression technology

The development of high-level hepatitis B vaccine production in *S. cerevisiae* illustrated some of the subtleties of gene regulation and heterologous protein expression – aspects of which can still confound biotechnologists today. It also marked the first commercial exploitation of gene expression in a eukaryotic host, and a new era in vaccine development. However, above and beyond expression technology, the greatest impact of this billion-dollar protein has been the protection it affords millions of individuals against a debilitating, often deadly, disease.

6.6 Further biotechnological applications of expression technology

Quite apart from producing proteins of commercial value, protein expression can be manipulated in *S. cerevisiae* to provide *in vivo* tools with which to probe molecular interactions. These sophisticated heterologous protein expression systems re-engineer promoter elements, transcription factors and signal cascade proteins to transduce heterologous molecular interactions into easily scoreable phenotypes. Such assays enable biotechnologists to screen for molecules that interfere with/enhance these interactions – so-called lead molecules in drug development. Reporter genes and growth on selective media have been used to examine the molecular biology of expressed heterologous steroid and peptide receptor proteins respectively, whereas the elegant two-hybrid technique, which exploits the modular nature of transcription factors, provides a window onto intracellular interactions between proteins.

6.6.1 Expression and analysis of heterologous receptor proteins

Oestrogen is an important human hormone that has been linked to breast cancer. As with many steroid hormones, oestrogen affects gene expression by binding to a cytoplasmically sequestered receptor protein and this complex then enters the cells nucleus, where it binds to promoters containing a specific receptor recognition sequence referred to as the oestrogen receptor element (ERE). Normal yeast cells do not contain either the receptor or ERE sequences. However, yeast cells have been re-engineered so that a β-galactosidase gene fused to a disabled *CYC1* promoter carrying the oestrogen receptor element (ERE) resides at the *URA3* chromosomal locus (Figure 6.7(a)). The same cells also carry a plasmid constitutively expressing the receptor protein intracellularly (Figure 6.7(b)). When these cells are now treated with oestrogen, the hormone

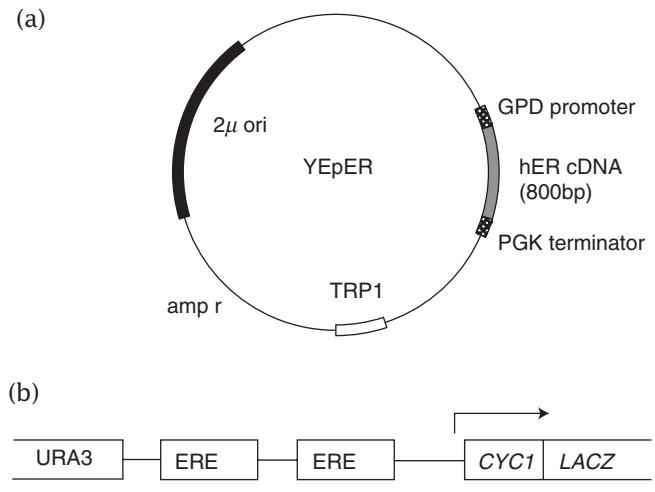


Figure 6.7 Analysis of oestrogen receptor protein

binds to the receptor and enters the nucleus, where it then binds to the ERE cloned into the promoter in front of the β-galactosidase gene, thereby inducing expression. This enzyme can be easily assayed. The level of enzyme detected in the cells is an index of oestrogen-induced receptor activity, allowing antagonists to be tested on the cells to identify which ones are best at inhibiting the hormone's activity. This reporter system was sufficiently sensitive to analyse the effect of site-specific mutations on hormone binding efficiency and to measure the effectiveness of agonists and antagonists on hormone action.

Unlike steroid hormone receptors, which when activated by hormone bind directly to promoter elements in the DNA, peptide hormones bind to receptor proteins that are embedded in the cell membrane. These molecular interactions are then communicated to the nucleus by kinase cascades (enzymes that add phosphate groups to proteins), ultimately resulting in the phosphorylation of a transcription factor, which induces gene expression via specific promoter elements. This type of signal transduction is controlled by so-called G-proteins (a heterotrimeric protein complex activated when a constitutively bound GDP molecule is replaced by a GTP molecule) that reside in the membrane next to the receptors. The latter translocate to yet another membrane protein that then initiates the kinase cascade – ending in the phosphorylation of a specific transcription factor and activation of gene expression. The precise structure of the receptors and G proteins, and the TF/promoter elements that they influence, varies from one organism to the next. Nevertheless, this overall cellular strategy for the transduction of a membrane signal into altered gene expression is highly conserved. It is for this reason that the extremely well characterized mating-signal-transduction pathway of *S. cerevisiae* can be re-engineered to

analyse the interactions of human peptides and their target membrane receptors (Figure 6.8).

Haploid cells secrete small peptides, which bind to receptors in the membranes of cells of the opposite mating type in order to synchronize their cell cycles for mating. The binding of α-factor from α-cells to the receptor protein of a normal haploid cell of 'a' mating type causes the activation of a G-protein in close contact with the receptor. The resulting kinase cascade causes a number of alterations in the cell's gene expression pattern. These include the activation of genes to arrest the cell in G1 of the cell cycle, and the expression of gene products ready for cell and then nuclear fusion as the synchronized cells mate together. With a view to using yeast cells in a high-throughput screening format, yeast cells have been manipulated to provide a 'read-out' of cell growth in a selective medium. The success of this extremely elegant approach to developing tools for screening drugs hinges on the fact that the mating pathway of yeast cells of 'a' mating type could be re-engineered such that they

- expressed a human receptor protein (SST_2) instead of the α-factor receptor
- expressed the α-sub-unit of the G-protein re-engineered so it could interact with the *human receptor protein* whilst retaining the segment that interacted with the yeast sub-units
- carried a *HIS3* wild-type gene driven by a promoter sensitive to activation by the TF normally activated by the mating pathway kinase cascade and
- carried a deleted *FAR1* gene to prevent cell cycle arrest when the kinase cascade is activated.

These cells no longer respond to the addition of α-factor because they lack the α-factor receptor protein. However, when the appropriate human peptide (somatostatin) is added to the cells, it binds to the heterologous human receptor protein in the membrane (Figure 6.8). The conformational change this causes is detected by the 'humanized' α-sub-unit of the G-protein. When GTP replaces GDP in this sub-unit, the sub-units dissociate, thereby initiating the kinase signal cascade to the nucleus. The activated transcription factor binds to the promoters of the genes normally induced by α-factor and to the promoter driving the *HIS3* gene product, thereby conferring a HIS+ phenotype on the cell. *Far1* activation would normally ensure that the cells arrest in G1 of the cell cycle, but as it has been deleted from this strain the cells progress through G1, allowing them to divide in His- selective medium.

In short, this re-engineered yeast cell transduces the heterologous human receptor-agonist interaction into a scoreable HIS+ phenotype. As G-protein-coupled receptors represent the targets for the majority of currently prescribed pharmaceutical drugs, this system has exciting potential for the development of high-throughput screening technology.

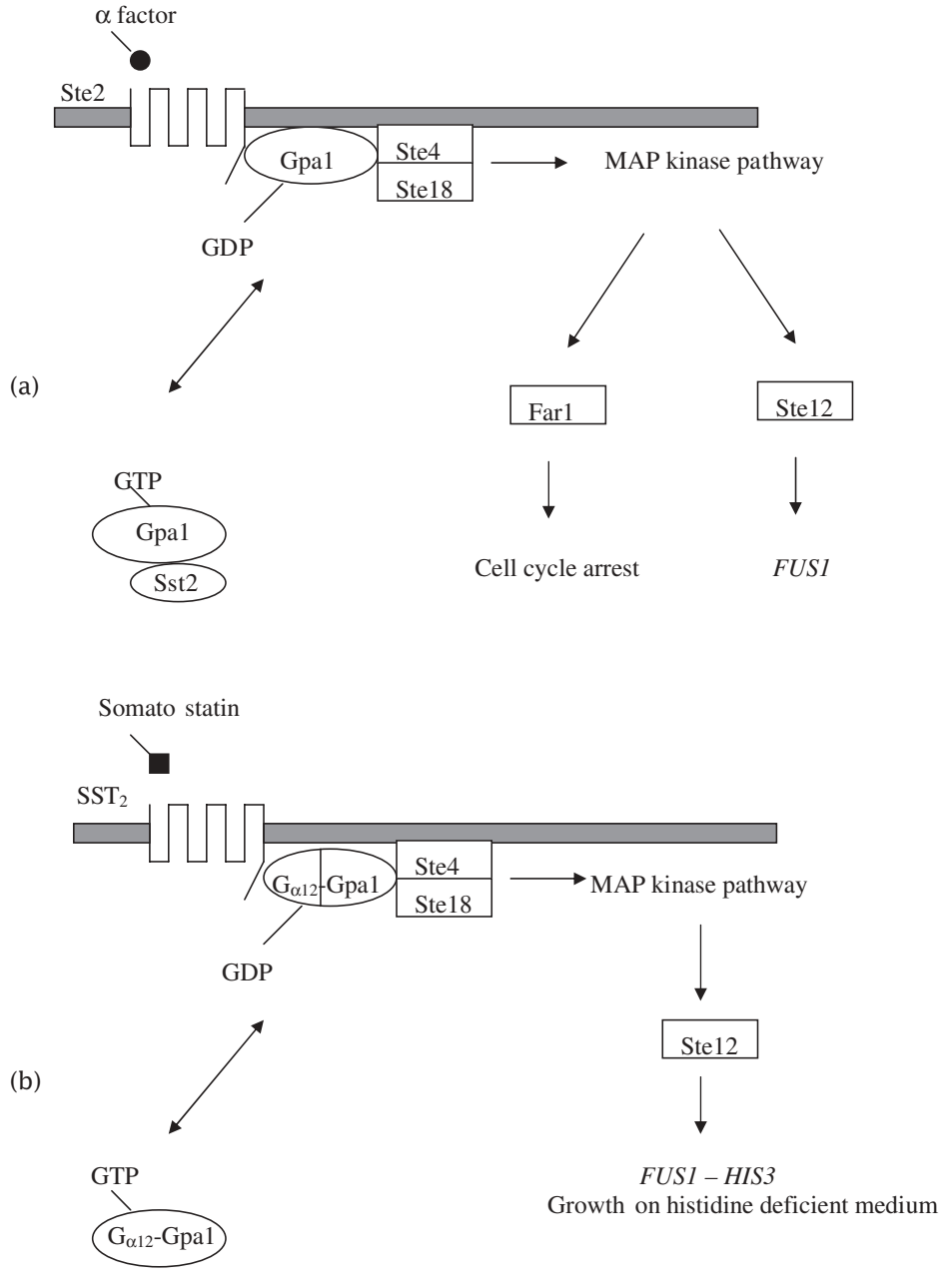


Figure 6.8 Heterologous receptor analysis using a re-engineered yeast pathway

6.6.2 2μ-hybrid analysis: an expression system that turns protein–protein interactions into a scoreable phenotype

This extremely clever expression system hinges on the fact that transcription factors consist of two separate domains: the DNA-binding (DBD) and activation (AD) domains. When expressed separately from different expression vectors, the two domains cannot interact and therefore a functional TF cannot be constructed. However, if the domains are expressed as fusion proteins with two proteins (X and Y) and the two proteins interact with one another in the cell, then the two TF domains are brought into contact with one another and a functional TF is regenerated. This can then drive gene expression (Figure 6.9).

Yeast vectors are available in which the latter DNA sequences that encode the DBD and AD of the yeast *GAL4* transcription factor (TF) are carried on separate expression plasmids. The DNA encoding one of the two potentially interacting proteins is fused with the sequence encoding the DBD; the DNA encoding the other potentially interacting protein is fused to the sequence encoding the AD. The plasmids are transformed into separate haploid cells carrying complementary genetic markers, defective in *HIS4*, and one of which has integrated into its chromosomal DNA a gene construct consisting of a GAL4-inducible promoter fused to the coding sequence for HIS4+. The two strains are then mated. If the two proteins interact, then this will bring the DBD and AD together in the cell, thereby reassembling the dissociated TF. Such cells will express the His4 gene product and be able to grow on selective His- medium (Figure 6.9). In cases where the expressed fusion proteins do not interact the two TF domains remain apart and although the DBD can bind to the His4 promoter it cannot activate transcription because it lacks the AD. Such a diploid

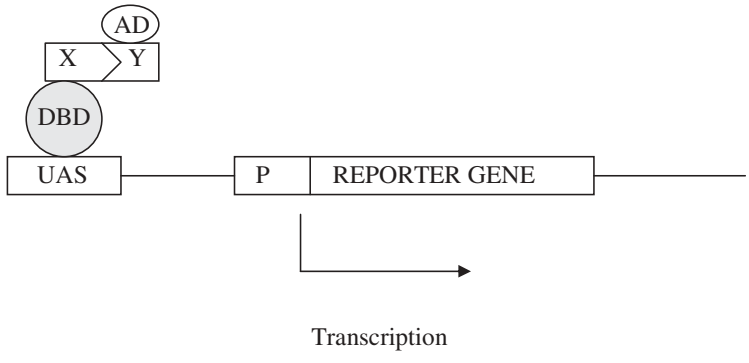


Figure 6.9 The two-hybrid system – only when 'X' interacts with 'Y' can the reporter gene get activated

strain will be unable to express *HIS4* and will therefore not grow on the selective medium. Not only has this so-called yeast two-hybrid system been successfully applied to study a broad spectrum of protein–protein interactions from many different species, it can also be used to detect small molecules that interfere with the targeted protein–protein interaction thereby providing yet another tool for drug discovery.

6.7 Conclusion

Ever since the successful expression of a hepatitis B vaccine in *S. cerevisiae*, yeasts have been key players in the biotechnology of heterologous protein production. This, coupled with their development as heterologous signal transduction systems, is set to continue into the foreseeable future. The early promise in this respect of their filamentous cousins; on the other hand, has yet to be realized. However, given that the protease problems can now be addressed, and that that the patents held on contemporarily important expression systems can be circumvented by using alternative hosts, the scene is now set for filamentous fungi to challenge the supremacy held heretofore by their simpler, single-celled, cousins.

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6.9 Revision questions

Q 6.1 Why are recombinant proteins much more difficult to genetically engineer than recombinant DNA molecules?

Q 6.2 Why is heterologous cDNA, rather than genomic DNA, normally used in expression vectors?

Q 6.3 List the important features of an expression vector.

Q 6.4 List three different types of DNA transformation procedures used in fungi.

Q 6.5 Why was *S. cerevisiae* the first eukaryotic heterologous expression system?

Q 6.6 Compare and contrast heterologous protein expression vector systems in *P. pastoris* and *S. cerevisiae*.

Q 6.7 Outline the important parameters that impact on successful heterologous protein production.

Q 6.8 Why was it not possible to use *E. coli* as the expression system for the hepatitis B vaccine?

Q 6.9 Compare and contrast the plasmids used as proof of principle and scale-up of the hepatitis B vaccine.

Q 6.10 Outline the cloning strategy used to use *S. cerevisiae* as a surrogate oestrogen receptor protein testing system.

Q 6.11 Outline the cloning strategy used to use *S. cerevisiae* as a surrogate G-protein signal transduction testing system.

Q 6.12 Explain the basis of the yeast two-hybrid system.

7
Fungal Diseases of Humans

Derek Sullivan, Gary Moran and David Coleman

7.1 Introduction

The ubiquity of fungi in the environment has already been described in earlier chapters. Fungal species have evolved and adapted to live in a wide variety of environments and ecological niches and consequently constitute a very diverse group of organisms. Fewer than 100 000 species of fungi have been identified to date; however, this is likely to be just the tip of the iceberg and it has been conservatively estimated that there are probably at least 1.5 million fungal species inhabiting our planet. Given this vast number of fungi and their prevalence in the environment, it is hardly surprising that humans come into contact with many hundreds of species of fungi every day and that in some cases humans have become potential sources of nutrients for some fungal species. For the most part, transient exposure to fungi or fungal colonization occurs without the knowledge of the affected individual. This is primarily due to the inherent low virulence of most fungi, especially when confronted with the full arsenal of the human immune system. However, some species of fungi are long established members of the human microflora (i.e. commensals), while under certain conditions some fungi can cause symptoms of disease and even death. As many as 200 fungal species have been associated with human infections (known as mycoses); however, fungi are far more important pathogens of plants and insects.

Some of the most common infections in humans are associated with fungi (e.g. thrush, dandruff and 'athlete's foot'). These are superficial and relatively innocuous; however, fungi can also cause far more devastating diseases, such as invasive aspergillosis and systemic candidosis, both of which have very high

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associated mortality rates. The incidence of these latter infections has been increasing in recent years and this has fuelled an enhanced interest amongst the clinical and scientific communities in mycoses and the fungal species responsible for them. Therefore, the purpose of this chapter is to introduce the reader to these diseases and to their etiological agents.

7.2 Fungal diseases

The types of infection caused by fungi can be classified in a number of ways. One division is based on whether the infection occurs in an otherwise healthy host or whether the host has an underlying medical condition causing impaired immune function (e.g. *opportunistic* infections). Another is based on whether the infection is confined to the outer layers of the epithelia or whether the infecting organisms penetrate through this barrier into the bloodstream and disseminate throughout the body. The former are referred to as *superficial* infections and the latter are referred to as *disseminated* infections (also called systemic or deep-seated infections).

7.3 Superficial mycoses

The human body is covered by skin, hair and nails, which given their location in the body are continuously exposed to the environment and a wide variety of environmental microbes. For the most part the keratinized epithelia that comprise the outer layers of the skin constitute an effective barrier, which excludes micro-organisms from gaining entry to deeper tissues. In addition, the skin also produces secretions, including sweat, sebum, transferrin and antimicrobial peptides known as defensins, which have the ability to kill many bacterial and fungal species. The skin is also equipped with intra-epithelial T and B cells as well as a range of phagocytes. However, a small number of fungal species have evolved mechanisms of overcoming these defensive mechanisms and can actively colonize the skin surface, becoming established as members of the normal skin microbial flora. From time to time (mainly for reasons that are still unclear), these fungi can cause symptoms of disease (see Table 7.1). Two examples of such infections are pityriasis versicolor and tinea nigra. The former is caused by a yeastlike organism known as *Malassezia furfur*. This organism thrives on the fatty acids found in sebum and affects pigment-producing cells, resulting in a pink rash on pale skin and hypopigmentation in darker skin. Interestingly, this species has also been associated with dandruff, a common ailment characterized by increased shedding of skin cells from the scalp. Tinea nigra, a rare disease characterized by a rash caused by the mould species *Hortaea werneckii* (previously known as *Exophiala werneckii*), results from the production of melanin by the fungus, which causes the formation of brown patches on the palms and

Table 7.1 Examples of superficial mycoses

Site of infection	Disease name	Causative agent
Skin	Pityriasis versicolor	*Malassezia furfur*
	Tinea nigra	*Hortaea werneckii*
	Ringworm (e.g. tinea capitis)	*Trichosporon and Microsporon* spp.
	Athlete's foot (i.e. tinea pedis)	*Trichosporon and Microsporon* spp.
Hair	White piedra	*Trichosporon beiglеii*
Nail	Tinea unguium	*Trichophyton rubrum*
Subcutaneous	Chromoblastomycosis	*Fonsecaea* spp.
	Sporotrichosis	*Sporothrix schenkii*
	Mycetoma	*Pseudallescheria boydii*

soles of the feet. As well as infecting the skin, fungi can also infect hair and nails. For example, the fungus *Trichosporon beigelii* causes a disease in hair known as white piedra, while *Trichophyton rubrum* causes the nail infection tinea unguium.

Due to the confinement of these infections to the extreme outer layers of the body there generally is no cellular immune response to the pathogens responsible for the disease. However, if the infecting fungi penetrate deep enough into the tissues to elicit an immune response the infections are sometimes referred to as cutaneous mycoses. The most important examples of these infections are known collectively as tinea (more commonly known as ringworm). These infections can occur in various locations in the body, ranging from the feet (tinea pedis, better known as athlete's foot) to the head (tinea capitis) and are caused by keratin-degrading fungi collectively known as the dermatophytes, which includes species such as *Trichophyton* spp. and *Microsporum* spp. These infections are usually self-limiting and can be treated relatively easily using topical and sometimes oral antifungal drugs, such as members of the azole family and terbinafine.

Rarely, fungi manage to penetrate deeper through the epidermis and cause infection in the underlying subcutaneous tissues (sometimes penetrating as deep as underlying bone). These infections are usually the result of the fungus gaining access to these tissues following trauma (e.g. wounds, splinters and bites). Examples of these infections are chromoblastomycosis, sporotrichosis and mycetoma.

7.4 Opportunistic mycoses

As already mentioned the innate and adaptive human immune systems are remarkably adept at protecting the human body from infection by fungi. Consequently, in normal healthy individuals fungal infections are relatively uncom-

Table 7.2 Examples of risk factors for opportunistic fungal infections

HIV infection and AIDS
Solid-organ transplantation
Anti-cancer chemotherapy
Granulocytopenia
Premature birth
Old age
Use of corticosteroids
Use of broad-spectrum antibiotics
Central vascular catheters
Gastrointestinal surgery
Colonization with fungus (e.g. *Candida* spp.)

Table 7.3 Examples of opportunistic mycoses

Causative agent	Disease
Candida spp.	Oral candidosis/denture stomatitis
	Vulvovaginal candidosis
	Systemic candidosis
Aspergillus spp.	Invasive aspergillosis
	Aspergilloma
	Allergic bronchopulmonary aspergillosis
Cryptococcus neoformans	Cryptococcal meningitis
Pneumocystis jiroveci	Pneumocystis pneumonia

mon. However, there is a wide range of clinical circumstances (see Table 7.2) that can result in certain fungal species causing disease. These infections are known as opportunistic mycoses, due to the fact that these fungi usually only cause disease in cases where patients' defence systems are not functioning adequately (see Table 7.3). The two most important opportunistic fungal pathogens are yeast species belonging to the genus *Candida* (which cause candidosis) and moulds belonging to the genus *Aspergillus* (which cause aspergillosis).

7.4.1 Candidosis

The genus *Candida* is comprised of approximately 200 yeast species, most of which have no known teleomorphic (i.e. sexual) phase. They are ubiquitous in the environment (often associated with plants and animals); however, approxi-

Table 7.4 *Candida* species associated with human disease

Candida albicans
Candida glabrata
Candida parapsilosis
Candida tropicalis
Candida dubliniensis
Candida krusei
Candida guilliermondii
Candida lusitaniae
Candida kefyr
Candida norvegensis
Candida famata
Candida inconspicua

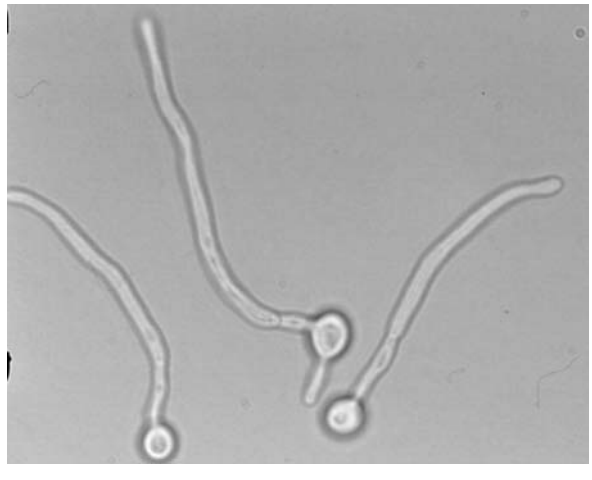

Figure 7.1 A photomicrograph of *C. albicans* cells (blastospores) producing hyphae

mately a dozen (see Table 7.4) have been associated with human commensalism or disease. These *Candida* species are carried innocuously by a large proportion of humans, particularly on the epithelial surfaces of the mouth, gastro-intestinal tract (GIT), vagina and skin. They typically grow as ovoid blastospores; however, under specific conditions most can produce filamentous cells known as pseudohyphae, while *Candida albicans* and *Candida dubliniensis* can produce true mycelium and refractile sporelike structures known as chlamydospores (see Figure 7.1). While these species are harmless in healthy individuals, when the host's immune defenses are compromised in any way these

organisms have the potential to overgrow and cause severe problems. Predisposing factors to candidosis include immuno-suppression (e.g. due to HIV infection, anticancer therapy and treatment with immuno-suppressive drugs used in organ transplantation), catheterization (which allows the direct inoculation of the yeast cells into tissue and blood vessels), premature birth (immature immune system), extreme old age (defective immune system), use of broad-spectrum antibiotics (disruption of the normal bacterial microflora), use of corticosteroids (disruption of local immune response), GIT surgery (direct inoculation of yeast cells into the blood stream) and prior colonization with *Candida* spp. (most cases of candidosis are acquired endogenously from the patient's normal microbial flora) (see Table 7.2). Once *Candida* cells have overcome the (impaired) immune response they can cause a wide range of infections. These range from superficial infections of the skin and the mucous membranes of the oral cavity and the vagina to cases when the cells penetrate through the epithelia and are disseminated throughout the body by the blood to infect a wide variety of organs including the kidney, liver and brain.

One of the most common fungal infections is vulvovaginal candidosis (VVC), an infection of the vulva and vaginal area, also known as thrush. It occurs in apparently normal healthy women, approximately 75 per cent of whom will become infected at least once during their reproductive years. The symptoms of the disease include discomfort, itching, erythema and the production of a whitish discharge. While VVC is usually easily treated using topical antifungal agents a small proportion of women suffer from recurrent infections which are particularly recalcitrant to conventional therapies. The predisposing factors for VVC are not clear; however, an association with pregnancy, diabetes, antibiotic use and HIV infection have been suggested.

Candida species are also associated with infections of the mucosal tissues of the oropharynx. These infections are known as oropharyngeal candidosis (OPC) and only occur in individuals whose immune systems have been compromised, such as those infected with HIV, neonates with immature immune systems, patients receiving steroid therapy for asthma and patients receiving head and neck radiotherapy therapy for the treatment of cancer. In addition, patients wearing dentures who practice poor oral hygiene can present with overgrowth of *Candida* spp. and inflammation of oral tissues in contact with the denture (i.e. denture stomatitis). There are several forms of OPC (see Plate 4), the most common of which is known as pseudomembranous candidosis (more commonly known as oral thrush). This is characterized by the presence of creamy-white patches (comprised of buccal cells, host protein exudate and candidal yeasts and hyphae) and overlying red patches primarily found on the palate and dorsum of the tongue. Other forms of OPC include erythematous candidosis and angular cheilitis. OPC causes a high degree of discomfort, particularly when eating and swallowing. It was originally treated using topical agents such as nystatin lozenges; however, this has largely been replaced by treatment with oral azole drugs, especially fluconazole. Recurrent infections can also occur in specific

patient populations and in these cases amphotericin B is often the most effective treatment option. OPC is most commonly observed in individuals infected with HIV. The vast majority of AIDS patients suffer from oral candidosis at some stage during their infection, and in the early stages of the AIDS epidemic OPC was often used as one of the diagnostic markers for HIV infection. On occasion, the infection can extend into the oesophagus, resulting in oesophageal candidosis. Fortunately, the introduction of highly active antiretroviral therapy (HAART) for the treatment of AIDS in the developed world has led to a significant reduction in the incidence of OPC in HIV-infected individuals.

Candida spp. can also cause infections on the skin (e.g. nappy rash and intertrigo). In addition to these infections, *Candida* spp. can also cause a more severe skin disease that is known as chronic mucocutaneous candidosis. This infection can occur in HIV-infected patients, individuals with endocrine and immune dysfunction and individuals with certain types of cancer, such as thymoma.

The infections described so far are superficial and confined to the outer layers of the skin and mucosal surfaces. However, under certain rare circumstances *Candida* cells can sometimes penetrate through this barrier, eventually reaching the bloodstream, thus causing candidaemia and ultimately resulting in disseminated infections in a wide range of organs (e.g. kidney, spleen liver) and systems (e.g. urinary tract). Invasive candidosis usually occurs in patients with severe neutropaenia (i.e. patients with depleted numbers of neutrophils, often due to treatment of cancer and haematological malignancy), patients who have undergone major abdominal surgery, patients receiving prolonged broad-spectrum antibacterial therapy and in patients with catheters (see Table 7.2). Consequently, these infections are usually only encountered in hospitalized individuals who are already very ill. How the yeast cells gain access to the bloodstream is not entirely clear. However, antineoplastic chemotherapy can damage the epithelia of the gastro-intestinal tract (GIT) sufficiently to allow the yeasts to translocate across the gut wall into the local blood vessels. Similarly, intestinal surgery and catheters can allow the yeasts direct access to the bloodstream. Under most circumstances the human immune system can eradicate low numbers of invading candidal cells (transient candidaemia); however, in patients with reduced numbers of neutrophils, the yeasts are allowed to overgrow and spread throughout the body. The symptoms associated with invasive candidosis are very difficult to discriminate from those of infections caused by other pathogens. One of the earliest signs is persistent fever that does not respond to broad-spectrum antibacterial therapy; however, in some cases skin lesions can also appear. If left untreated candidaemia can result in candidal cells being distributed to organs such as the liver, kidney, liver and brain, ultimately leading to death. The crude mortality due to candidal blood infections is often greater than 40 per cent and it has been estimated that in the USA alone the attributable costs associated with candidaemia may be as high as one billion dollars per year. Invasive candidosis is usually treated with azole and polyene drugs (especially lipid formulations of amphotericin B). In addition, novel azoles (e.g.

voriconazole) and the new class of drugs known as the echinocandins offer the chance of improved survival rates in infected patients.

Diagnosis and epidemiology of Candida *infections*

In order to effectively treat candidal infections it is imperative that the yeast is detected and identified as rapidly as possible. In the case of superficial infections, such as VVC and OPC, this is relatively straightforward. Swabs can be taken from the affected area and inoculated onto routine mycological agar plates. The chromogenic medium CHROMagar Candida™ has been shown to be particularly useful in enumerating and identifying the yeast species present, with different species of *Candida* yielding colonies with characteristic colours (see Plate 5). However, the diagnosis of candidaemia is more problematic. As mentioned earlier, the symptoms of candidaemia are non-specific, and could easily be confused with other bloodstream infections. When candidaemia is suspected, a blood sample should be taken and inoculated into culture medium and analysed using manual or automated culture methods. However, in some cases it can take up to 48 hours for growth to be detected and it has also been reported that in many cases of candidaemia blood culture fails to detect evidence of *Candida*, mainly due to the relatively low levels of *Candida* cells usually present in the blood. This can result in a delay in the provision of appropriate therapy and increased mortality. Other tests to detect *Candida* spp. in blood include the detection of anti-*Candida* antibodies and *Candida* antigens in blood samples. In order to improve the speed, sensitivity and specificity of fungal infection diagnosis, molecular methods based on the polymerase chain reaction (PCR) are currently being assessed. This test relies on the detection and amplification of very small amounts of candidal DNA in blood samples using the thermostable enzyme *Taq* polymerase. PCR is exquisitely sensitive (it can detect as few as ten *Candida* cells per millilitre of blood) and results can be obtained within a few hours. However, the exquisite sensitivity of PCR can lead to problems, with false positive results occurring due to problems associated with contamination and the detection of non-viable cells.

The epidemiology of candidosis is constantly changing and mirrors developments in other aspects of human disease. The incidence of superficial forms of the disease, particularly OPC, increased dramatically during the 1980s and 1990s. This increase was due primarily to the HIV pandemic, which began in the 1980s and which resulted in large numbers of individuals at risk of infection. However, the introduction in the late 1990s of combination therapies, including novel anti-HIV agents such as protease inhibitors, which lead to a reconstitution of the T helper cell count, has resulted in a marked decline in the prevalence of OPC in developed countries. Despite this, candidal infections can still occur in HIV-infected patients in whom HAART has failed or in cases where the patient does not follow the full course of therapy (i.e non-compliance).

Unfortunately, due to their high cost these therapies are rarely provided in underdeveloped countries around the world, where the HIV pandemic continues unabated.

Due to the increased number of individuals at risk, the incidence of invasive candidosis has also increased in recent years and is now believed to be the fourth most common cause of nosocomially acquired (hospital-based) bloodstream infections (the leading causes are Gram-positive bacteria such as *Staphylococcus aureus* and *Staphylococcus epidermidis*). Recent studies have determined the incidence of candidaemia in two US regions to be approximately 8/100 000 population/year. The most likely reasons for the increased prevalence of candidal systemic infection are the development of more aggressive anti-cancer therapies and more powerful immunosuppressive drugs associated with organ transplantation. These have resulted in a greater number of individuals surviving life-threatening diseases; however, these patients are usually extremely ill and are immunocompromised for increasingly long periods of time. Consequently, the increased number of these patients provides an ever-growing population of at-risk individuals in our hospitals, who are very susceptible to fungal infection. In order to protect patients from these infections and to decrease the incidence of systemic candidal disease, many hospitals treat their patients prophylactically with antifungal drugs.

The *Candida* species most frequently associated with human infection is *Candida albicans*. This species is widely regarded as being the most important human yeast pathogen and when people refer to *Candida* it is often assumed that the species in question is *C. albicans*. However, in many epidemiological studies it is clear that *Candida* species other than *C. albicans* can cause both superficial and systemic disease (see Table 7.4). The exact proportions of species responsible for human disease can vary from country to country and even between different hospitals in the same geographical region. In the majority of studies *C. albicans* is the most commonly identified species in clinical samples. However, species such as *C. glabrata* and *C. parapsilosis* are often also identified as contributors to human disease. The reasons for the apparent increased prevalence of these species are not clear. However, since *C. glabrata* can rapidly develop resistance to antifungal drugs it has been suggested that prior treatment with these drugs can select for infections caused by this species. In addition, *C. parapsilosis* is often found in biofilms growing on plastic surfaces and has been primarily associated with infections resulting from procedures requiring catheterization.

The fact that some species are inherently resistant (e.g. *Candida krusei*) or can develop resistance (e.g. *C. glabrata*) to many antifungal drugs has significant implications for the choice of antifungal therapy. Therefore, in order to prescribe the optimum therapy it is important to be able to identify the species responsible for a particular infection as rapidly as possible. Unfortunately, it is quite difficult to readily discriminate between different *Candida* species. Their cell and colony morphologies are very similar, with the cells of most species

being similar in size and shape, while colonies cultured on routine diagnostic agar (such as Sabouraud's dextrose agar) are often very similar in size, shape, texture and colour. However, as mentioned earlier, the chromogenic agar medium CHROMagar Candida has been shown to be a useful aid in species identification, with the most clinically important *Candida* species being distinguishable on the basis of colony colour. In diagnostic mycology laboratories there are two 'gold standard' tests commonly used for the identification of *C. albicans*. These rely on the fact that *C. albicans* produces germ tubes (the first stage of hyphal development as the hyphae emerge from the cell) when incubated in serum and chlamydospores (thick walled refractile spores of unknown function or importance) when cultured on particular nutrient-depleted media. Until 1995, *C. albicans* was the only species known to produce germ tubes and chlamydospores; however, in 1995, a novel, closely related species, *Candida dubliniensis*, was identified in HIV-infected individuals, which can also exhibit these two morphological characteristics. Other tests used routinely to identify specific *Candida* species include the analysis of carbohydrate assimilation profiles and serological tests. Molecular tests, in particular those based on PCR, have also been developed to allow the rapid identification of specific species and offer great hope for future diagnostic tests.

While it is certainly very important to be able to discriminate between *Candida* species, for epidemiological studies it is often also very useful to be able to distinguish between strains within a species. This is particularly important when investigating the source of an infection and in determining whether a recurrent infection is due to re-infection with the original or a new strain or whether more than one strain is present in a clinical sample, all of which can have a significant effect on the course of therapy for an infection. A wide variety of methods of differentiating between strains have been developed. The earliest methods used were based on the comparison of phenotypic characteristics (e.g. sugar assimilation, colony morphology, resistance profiles); however, these tests have poor discriminatory abilities and have largely been superseded by molecular methods, which are far more discriminatory. Molecular strain typing methods are mainly based on the comparisons of the genetic content of individual strains. The most commonly used method is DNA fingerprinting using species-specific probes homologous to regions of DNA repeated in the candidal genome. This generates a bar-code-like pattern, which is strain specific and, with the aid of computers, can be compared quantitatively to similar patterns obtained for other strains. The karyotype (chromosome content) and the electrophoretic mobility of certain enzymes can also be used as a means of strain comparison. These methods have been applied widely to investigate the origin of *Candida* infections. In the case of the majority of superficial and systemic infections, it appears that the infecting strains are mainly acquired endogenously from the patients' own colonizing flora, confirming the observation that prior colonization with *Candida* is a risk factor for candidosis. However, there are numerous cases in the literature describing the identification of strains present

in a patient and on environmental surfaces and on the hands of health-care workers, suggesting that in some cases of disease the source of infection may be acquired from an exogenous source.

Candidal virulence factors

Since *Candida* species mainly cause disease in immunocompromised individuals, it would be easy to conclude that the most important factors contributing to the establishment of such infections are purely related to the host. While host factors undoubtedly play a significant role in the development of candidosis, traits associated with the yeast are also very important. The capacity of a microorganism to cause disease in a host is known as its virulence. Clearly, certain microbes are more predisposed to cause disease than others, and this is usually due to the possession of specific attributes related to the ability to cause damage to host tissue. These attributes are often referred to collectively as virulence factors. In the case of some bacterial species, it is very easy to identify virulence factors (e.g. the toxins produced by *Vibrio cholerae* and *Clostridium botulinum*); however, in the case of *Candida* species, it is quite difficult to establish which characteristics contribute to pathogenesis. Since *C. albicans* remains the most important yeast pathogen, its virulence mechanisms have been studied far more intensively than any other *Candida* species. From these studies, a number of traits expressed by *C. albicans* have been identified as being putative virulence factors (Table 7.5).

One of the most important requirements for any microbe, whether commensal or pathogenic, is the ability to adhere to host tissue. This requires that the microbial cell be able to recognize and to stick to host ligands, such as extracellular matrix proteins and cell membrane components. This allows the organisms to establish a foothold on the host surface, which prevents it from being dislodged by host secretions, such as saliva or sweat. So far a number of such proteins have been identified in *C. albicans*. Adhesins have been identified that bind to a range of host proteins, such as fibronectin and components of complement, as well as to host cell surface carbohydrate moieties of membrane glycolipids and glycoproteins. In addition, a large family of related proteins, known as the ALS (alpha-agglutinin-like sequence) family, has been shown to be involved in yeast–host cell interactions. The surfaces of hyphal *C. albicans* cells

Table 7.5 Putative virulence factors of *C. albicans*

Adhesins (e.g. HWP1, ALS proteins)
Yeast ↔ hyphal dimorphism
Phenotypic switching
Extracellular hydrolases (e.g. proteinases and lipases)

have also been shown to express a protein known as hyphal wall protein (Hwp1), which appears to act as a substrate for host enzymes (transglutaminases) that can covalently link the hyphal Hwp1 proteins directly to proteins on the surfaces of the epithelial cells.

One of the most important phenotypic characteristics of *C. albicans* (and *C. dubliniensis*) is its ability to exist as ovoid blastospores and elongated hyphal filaments (i.e. dimorphism). Both of these forms have been observed in tissue samples and it is possible that each form may contribute to different stages or different types of candidal infection. A range of environmental conditions have been shown to induce hyphal formation (e.g. serum, growth temperature greater than 35 °C, nutrient starvation and pH greater than 6.5); however, the precise nature of the genetic switch(es) involved are complex and as yet not fully understood. Interestingly, several virulence factors have been associated with specific morphological forms (e.g. the adhesin Hwp1 and several aspartyl proteinases (see below)).

Another proposed virulence factor of *C. albicans* is known as phenotypic switching. When a single strain of *C. albicans* is plated out onto agar, unusual colony variants are often observed (at a frequency of usually around 1 in 1000). These colonies can have different shape, texture and/or colour and the altered colony architecture is usually due to alterations in the morphologies of the constituent cells. The switch event is reversible, suggesting the presence of a precise genetic switch responsible for the turning on and off of a wide range of unlinked genes. Switch types have been shown to differ in their ability to express a range of virulence factors (e.g. adhesins, proteinases and dimorphism) and, therefore, it has been suggested that phenotypic switching is a means to allow the asexual yeast to generate diversity to facilitate adaptation to growing in diverse environments and possibly to evade the host immune response. Of particular interest is the recent discovery that in order for *C. albicans* cells to be forced to mate they must exist in a particular switch phenotype.

As in the case of most other pathogens, *C. albicans* has been shown to produce a wide range of extracellular enzymes that can digest host proteins. In particular, *C. albicans* contains a family of ten closely related secreted aspartyl proteinases (SAPs) that have the ability to hydrolyse a wide range of host proteins, ranging from matrix components to immune system proteins such as antibodies. In addition, *C. albicans* also encodes a family of lipases and phospholipases that have also been implicated in virulence. Interestingly, specific proteinases and lipases are quite stringently regulated, only being expressed in specific morphogical or switch phenotypes.

Clearly, candidal pathogenesis is a very complicated and multifactorial process, with a vast array of different virulence factors interacting in specific microenvironments to contribute to the survival and proliferation of the organism. (See Plate 6 for a summary of events. (1) *Candida* cells adhere to the epithelial cell surface via specific interactions between candidal adhesins and host ligands. (2) Once they have bound to the tissue the cells begin to proliferate

and to produce hyphae. (3) Growth continues by budding and production of hyphae (growth is often associated with formation of a biofilm). (4) Fungal hyphae and cells eventually penetrate through the epithelial layer to the tissues below, ultimately reaching the bloodstream, via which they disseminate throughout the body to cause disseminated candidosis.) However, it is becoming clear that it is naïve to think of *Candida* cells acting in isolation. In nature it is now believed that the majority of microbes exist in complex communities of micro-organisms attached to surfaces and covered in extracellular matrix. These communities are known as biofilm, and it is now believed that that the ability to form biofilm is an integral component of the candidal offensive armoury. Candidal biofilms are of particular interest because it has been demonstrated that cells in biofilm are more tolerant of antifungal drugs. In addition to *C. albicans*, *C. parapsilosis* has also been demonstrated to form biofilm, particularly on the surfaces of plastic catheters, possibly explaining the high level of association between this species and catheter-related infections.

7.4.2 Aspergillosis

While certain yeast species are important human pathogens, some filamentous fungi and moulds also, under specific circumstances, have the ability to cause serious infections in humans. The most important of these moulds belong to the genus *Aspergillus*. Approximately 20 *Aspergillus* species have been associated with human infections; however, the vast majority of cases of aspergillosis are caused by only a handful of species, especially *A. fumigatus*, *A. niger*, *A. terreus* and *A. flavus*. These fungi are saprophytic and ubiquitous in the environment and are particularly associated with soil and decaying vegetable matter. They grow as a mass of branching hyphae; however, they also produce vast numbers of spores (known as conidia) in structures known as conidiophores (see Figure 7.2). Conidia are released into the environment and can be carried great distances by air currents. The concentration of conidia in air can range from 1 to

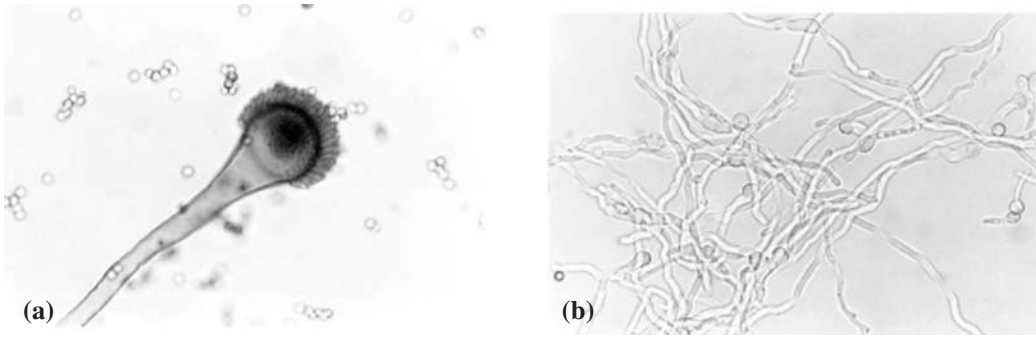

Figure 7.2 *Aspergillus fumigatus:* (a) conidiophore, (b) germinating conidia

100 per cubic metre. Therefore, they are routinely inhaled by humans and their size (approximately 2–3 μm) allows them to penetrate deep into the lower respiratory tract. In normal healthy individuals these are easily detected and destroyed by the innate pulmonary immune system; however, in certain subsets of the population the spores can settle and cause a wide range of diseases.

The most important form of aspergillosis is known as invasive aspergillosis (IA). Patients at risk of acquiring this infection are generally profoundly neutropenic for prolonged periods of time. Patients most at risk of acquiring IA include those receiving immunosuppressive treatment for organ transplantations (especially bone marrow transplants) and those receiving cytotoxic antineoplastic therapy. Reduced neutrophil counts and impaired alveolar macrophage function prevent the host from destroying *Aspergillus* conidia that are inhaled into the alveoli, allowing the conidia to germinate and the developing hyphae to proliferate. If not detected and treated in time the hyphae can eventually penetrate through the alveolar wall, before ultimately reaching and penetrating into the circulatory system. Fragments of hyphae can then be spread to a wide range of organs throughout the body, resulting in disseminated aspergillosis. The symptoms of IA are non-specific, but usually include fever and sometimes chest discomfort and cough (sometimes with blood). The mortality rate of IA is very high (50–100 per cent), especially once the fungi have spread to the other organs, particularly the brain. Clearly, early diagnosis of IA is essential to allow optimum treatment. However, this is very difficult to achieve as the available diagnostic methods are not very effective. Methods of diagnosis include histological analysis and culture of biopsy and broncoalveolar lavage fluid taken from the infected area of the lung. Radiography and serological and molecular (PCR) tests of blood samples can also be used. However, despite all of these tests definitive diagnosis IA is often only obtained at *post mortem*. Invasive aspergillosis is usually treated with liposomal amphotericin B; however, recently developed drugs, such as itraconazole, voriconazole and caspofungin, have also been shown to be effective. In addition, attempts can be made to minimize the risk of IA by using prophylactic doses of drug during periods of profound neutropaenia and by minimizing the risk of inhaling conidia by maintaining patients in rooms with filtered air.

Aspergillus spp. can also cause a range of other diseases, often in non-immunocompromised individuals. One example is aspergilloma, an infection which develops due to *Aspergillus* species colonizing areas in the lung that have been damaged (e.g. by tuberculosis and sarcoidosis scars and cavities). The fungus proliferates and forms a large ball-like hyphal mass (the aspergilloma). The infection is often asymptomatic; however, fever and coughing up blood (haemoptysis) can also develop. It is usually treatable using conventional antifungal drugs; however, in extreme cases the fungal ball has to be surgically excised. Another disease caused by *Aspergillus* species is allergic bronchopulmonary aspergillosis (ABPA). This is an allergy to toxic products produced by *Aspergillus* cells that chronically colonize the upper respiratory tract, especially in asthmatic and cystic fibrosis patients.

A wide range of moulds other than *Aspergillus* spp. have been identified as being potential human pathogens in severely immunocompromised individuals. These include the dematiaceous fungi (so called because of their dark colour) (e.g. *Alternaria* and *Pseudallescheria* spp.), *Fusarium* spp. and the zygomycetes, such as *Mucor* and *Rhizopus* spp. While infections caused by these species are still relatively rare, their incidence is increasing due to the growing numbers of patients with severely compromised immune systems for longer periods of time. It is particularly noteworthy that it is often difficult to identify many of these moulds in clinical samples, and in any case many of them are not susceptible to most of the commonly used antifungal drugs. Consequently, these mycoses have become a significant concern for clinicians.

7.4.3 Cryptococcosis

Cryptococcus neoformans is the most pathogenic *Cryptococcus* species. It is a sexually reproducing yeast, which produces a characteristic carbohydrate capsule (an important virulence factor) and is unable to produce hyphae. The natural reservoir of *C. neoformans* is believed to be the soil, particularly in areas with high levels of pigeon guano. Infectious propagules of the fungus are thought to be carried by air currents and are regularly inhaled by humans. In the majority of cases the fungi are cleared by the immune system or a transient non-symptomatic infection can sometimes occur. However, in susceptible individuals, especially HIV-infected and AIDS patients, the yeast cells can disseminate from the lungs into the bloodstream. Yeasts ultimately reach the central nervous system and the meninges, resulting in cryptococcal meningitis, a very serious infection with high mortality rates. The most common treatment is with a combination of amphotericin B and 5-fluorocytosine. While the levels of cryptococcosis were very high during the 1980s and 1990s, the introduction of HAART for the treatment of AIDS has resulted in a marked decrease in the incidence of the disease.

7.4.4 *Pneumocystis* pneumonia (PCP)

Pneumonia caused by the fungal species *Pneumocystis jiroveci* (until recently known as *Pneumocystis carinii* or *P. carinii* f. sp. *hominis*) is one of the most commonly encountered opportunistic infections associated with AIDS, although a wide range of other immunocompromised individuals can also acquire the disease. It has also been associated with outbreaks of infection in malnourished children in crowded institutions. *P. jiroveci* was originally considered to be a protozoan; however, in-depth phenotypic and genotypic analyses have confirmed it is more closely related to fungi. It is believed that this species is prevalent sub-clinically in the lungs of a large proportion of the human population and is either latent or is continuously encountered by individuals. In either

case, when the human T-cell count becomes depleted (e.g. by HIV infection or immunosuppressive therapy) the infection becomes activated, resulting in pneumonia-like symptoms, which if untreated can lead to death due to hypoxia. PCP is difficult to diagnose because *P. jiroveci* cannot be cultured *in vitro* and it is difficult to treat because *P. jiroveci* cell membranes do not contain ergosterol (the target of common antifungal drugs such as the azoles and amphotericin B). Consequently, the disease is treated using trimethoprim-sulfamethoxazole, which can have serious side-effects. Fortunately, due to the recent success of anti-HIV triple therapy, the incidence of PCP is decreasing.

7.5 Endemic systemic mycoses

Not all systemic fungal infections require an immunocompromised host. There are several examples of fungi that are primary pathogens (i.e. naturally virulent for humans) and that can cause symptoms of disease in individuals who are apparently otherwise healthy (Table 7.6). These infections are usually most often found in very specific geographic locations and hence are often referred to as endemic mycoses.

7.5.1 Histoplasmosis

Histoplasmosis is caused by the dimorphic fungus *Histoplasma capsulatum*. This sexually reproducing fungal species is naturally found in the soil (often associated with avian and bat guano) and is endemic in tropical areas of the world and in the Mississippi river basin in the USA. In the soil and at 25 °C in the laboratory the fungus exists in a hyphal form; however, in the human host and at 37 °C *in vitro* it exists as small (approximately 3 mm diameter) conidia. It is interesting to note that in *C. albicans* both the yeast and hyphal forms are thought to be important in virulence, while in *H. capsulatum* (and other systemic mycoses) only the conidial form is associated with disease. Inhalation of the infectious propagules by normal healthy individuals usually results in a self-limiting sub-clinical infection, although in a small number of cases the patient

Table 7.6 Examples of endemic systemic mycoses

Causative agent	Disease
Histoplasma capsulatum	Histoplasmosis
Blastomyces dermatitidis	Blastomycosis
Coccidioides immitis	Coccidioidomycosis
Paracoccidioides brasiliensis	Paracoccidioidomycosis

may complain of mild flulike symptoms. In cases where the patient happens to be immunocompromised (e.g. infected with HIV), histoplasmosis can be life threatening. An important virulence factor of *H. capsulatum* is the ability of this species to survive and proliferate within macrophages following phagocytosis. In certain circumstances, the *H. capsulatum* cells can remain latent and become reactivated in tissue years following the original exposure to the fungus, appearing once the immune system begins to deteriorate. Histoplasmosis hit the news headlines in 1997 when the singer Bob Dylan was hospitalized due to the illness.

7.5.2 Blastomycosis, coccidioidomycosis and paracoccidioidomycosis

The causative agent of blastomycosis, *Blastomyces dermatitidis*, is very similar to *H. capsulatum* in that it is also a sexually reproducing dimorphic fungus that can cause primary human infections. As with histoplasmosis, blastomycosis is acquired by inhaling conidia from contaminated damp soil and is endemic in large areas of North America. In the majority of cases the infection causes little or no symptoms; however, progressive pulmonary and/or systemic infection can rarely occur.

Coccidioides immitis, the causative agent of coccidioidomycosis (also known as Valley fever) grows in the hyphal form in its natural habitat (alkaline soils in dry and arid regions of the southwest USA and northern Mexico). The fungus produces sporelike structures known as arthroconidia, which when inhaled into the lungs of normal healthy individuals develop into multinucleate spherical structures, called spherules, that are filled with endospores. This results in respiratory infection, which is normally self-limiting, although infected individuals sometimes complain of cough and fever. However, in approximately 5 per cent of cases the disease can disseminate and progress to become a more serious disease. Another disease caused by a dimorphic fungus is paracoccidioidomycosis, which is caused by the dimorphic pathogen *Parracoccidioides brasiliensis*. This infection is endemic in Central and South America and as occurs with the other systemic mycoses results from the inhalation of spores from the soil. The primary disease is usually sub-clinical; however, for reasons as yet unknown, symptomatic infections are primarily diagnosed in males.

7.5.3 Mycotoxicoses

Like all organisms, fungi produce by-products of metabolism as they grow. The majority of these low-molecular-weight compounds are harmless to humans; however, some can deleteriously affect human health and these are referred to as mycotoxins. It should also be remembered that some fungal metabolites can

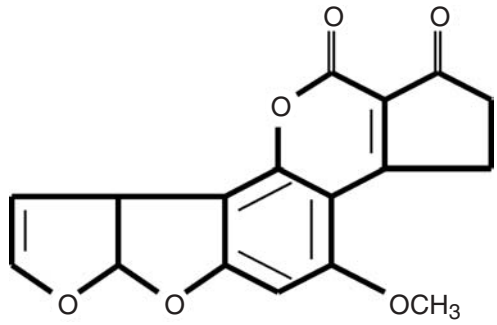

Figure 7.3 Diagram of the chemical structure of aflatoxin B_1

be beneficial to humans (e.g. antibiotics produced by fungi). While there are approximately 300–400 recognized mycotoxins, only ten or so are commonly observed in disease, which are collectively known as mycotoxicoses. The best known of mycotoxins are the aflatoxin family and the ergot alkaloids.

There are four major aflatoxins (B_1, B_2, G_1 and G_2); however, B_1 is the most important (see Figure 7.3). In biochemical terms the aflatoxins are difuranocoumarin derivatives and are produced by a range of aspergillus species, particularly *Aspergillus flavus*. These fungi frequently contaminate and produce toxins while growing on crops such as corn and peanuts, which are subsequently consumed by animals and humans. Consumption of meat and milk from cows that have been exposed to the mycotoxins can also result in exposure of humans to the toxins. While acute aflatoxicosis is a relatively rare phenomenon, ingested aflatoxins are mainly notorious for their carcinogenicity as a result of chronic exposure. The mutagenic nature of aflatoxins is believed to be due to the DNA-damaging properties of aflatoxin metabolic derivatives. The most common form of disease associated with dietary exposure to aflatoxins is liver cancer; however, it may also be implicated in other forms of cancer. Because *Aspergillus* species are ubiquitous in the environment it is impossible to prevent foodstuff contamination with fungi. However, this contamination can be minimized by the use of stringent production, storage and monitoring procedures.

Ergotism (also known as St. Anthony's fire) is a disease associated with the ingestion of cereals contaminated with the fungus *Claviceps purpurea*. This fungus, which infects the flowers of grasses and cereals, produces a range of alkaloids, of which ergotamine is the best known. Ergotamine is related to the hallucinogen LSD and ingestion of cereals and cereal-derived products such as rye bread that are contaminated with the fungus can result in serious symptoms of disease, such as convulsions and gangrene.

It has been suggested that mycotoxins might also exert a damaging effect through inhalation, rather than by ingestion. Most homes, office buildings and factories provide many niches suitable for the growth of a myriad of filamentous fungi, including *Aspergillus* spp., and *Claviceps* spp., and it has been sug-

gested that mycotoxins produced by these species might contribute to the phenomenon known as 'sick building syndrome'. This syndrome has been associated with an ill defined group of non-specific symptoms (usually including fatigue, minor respiratory problems and headaches) that are only experienced within a particular building. The aetiology of sick building syndrome is not known; however, poor air quality and ventilation, cleaning chemicals and microbial contamination have all been suggested as contributory causes. However, the role of fungi in the syndrome is a source of considerable conjecture.

Spores and volatile by-products from a wide range of filamentous fungal species can also act as allergens. Since most fungi come into contact with humans by inhalation into the respiratory tract, the symptoms of allergy usually occur in the sinuses and lungs. Symptoms are similar to those of other allergies such as hay fever and asthma, and are usually caused by IgE-mediated type I hypersensitivity reactions.

7.6 Concluding remarks

Considering the vast number of fungi that most of us come into contact with every day, fungal diseases are less prevalent than perhaps might be expected. The fact that fungal infections are relatively rare is testament to the amazing efficiency of the human immune system, and from the descriptions of the mycoses described above it is clear that when the immune system fails the fallout can be catastrophic for the patient. As great improvements continue to be made in medical science, especially in organ transplantation and cancer treatment, there will be an ever-increasing number of severely ill patients with profound immunosuppression for longer periods in our hospitals. Therefore, the number of individuals at risk of acquiring life-threatening fungal infections is growing all the time. Mycologists are becoming increasingly able to prevent, diagnose and treat these diseases, thus improving the prognosis for a wide range of patients.

Over the past 30 years, the prevalence of many fungal infections has changed dramatically for a wide variety of reasons. For instance, in the early 1980s it would have been impossible to forecast the huge increase (and subsequent decrease during the late 1990s) in the incidence of oral candidosis associated with AIDS. Therefore, it is essential that clinicians, epidemiologists and mycologists remain vigilant and are ready for future changes in the epidemiology of fungal diseases. Future medical advances, viral epidemics and bioterrorism could all alter the spectrum of fungal pathogens plaguing humans in the future.

7.7 Further reading

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7.8 Revision questions

Q 7.1 What are the most common superficial fungal infections and what are the causative agents?

Q 7.2 Name the four *Candida* species most commonly associated with human infections.

Q 7.3 What are the most important risk factors for opportunistic fungal infections?

Q 7.4 What are the most important virulence factors associated with the ability of *Candida albicans* to cause disease?

Q 7.5 What types of infection can be caused by *Aspergillus* species?

Q 7.6 By what name is the fungal species formerly known as *Pneumocystis carinii* now known?

Q 7.7 What is the most important form of aflatoxin and what are its deleterious effects on humans?

8

Antifungal Agents for Use in Human Therapy

Khaled H. Abu-Elteen and Mawieh Hamad

8.1 Introduction

Despite the successes and achievements of modern medicine, competent immunity remains the best hope for effective and long-lasting protection against infection and disease. Inherited and acquired causes, however, render the immune system deficient. The competence of the immune system can also be compromised by several disease states, chemotherapy and radiotherapy. Both immunocompromised and immunodeficient individuals are highly susceptible to viral, bacterial and fungal infections. The rising trends of viral, bacterial and fungal infections coupled with the appearance of new infective agents, the development of resistance against existing drugs and the toxic side-effects associated with current drugs necessitates a serious and continuous search for safer, more powerful and more selective anti-infective drugs.

With regard to fungal infections, potassium iodide was introduced in 1903 as an antifungal agent, followed by Whitefield's ointment in 1907 and then undecylenic acid in 1940. In the early 1950s, polyene antifungal drugs (nystatin and amphotericin B) were discovered and applied clinically. Since then, several classes of antifungal compounds have been introduced; however, serious problems continue to plague the use of antifungal compounds as therapeutic agents. Absence of selective toxicity of antifungal agents remains a major problem in the clinical application of these drugs. Fungi, like mammalian cells, are eukaryotic cells and as such there is a great resemblance in cell structure and metabolism between them. Therefore, antifungal agents can indiscriminately disrupt the normal metabolic processes of both fungal and host cells. This is why the

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development of toxic side-effects is always a concern during or following chemotherapy with antifungal drugs. Insolubility or decreased solubility of many antifungal agents and the poor absorption through the gastrointestinal tract demands the use of parenteral routes, which increases the levels of toxicity associated with the use of antifungal agents. Fungi tend to infect poorly vascularized tissues; poor tissue penetration and distribution of systemic antifungals adversely affect the therapeutic efficacy of antifungal compounds.

Great efforts and large sums of money have been, and are being, spent on the development of new antifungal agents; nonetheless, the number of antifungal agents approved for clinical use is very limited. In the USA, only ten antifungal compounds are FDA approved to treat fungal infections. The limited number of molecular targets that fungal agents can attack to kill or disrupt the metabolism of fungi makes the development of new drugs more challenging.

Antifungal agents currently available for clinical use belong to three major classes: polyenes, azoles and 5-fluorocytosine. Additional antifungal compounds such as allylamines, candines and nucleoside analogues plus some miscellaneous compounds with antifungal activity are also available. Optimally, antifungals should exhibit selective toxicity; they should be able to inhibit the growth of the fungus without adversely affecting the host. Based on the mechanism of action and the degree of toxicity to target fungal cells, antifungal agents can kill cells (fungicidal) or reversibly inhibit their growth (fungistatic). Antifungal agents may be naturally derived (antibiotic) or chemically synthesized (synthetic) compounds.

8.2 Polyene antifungal agents

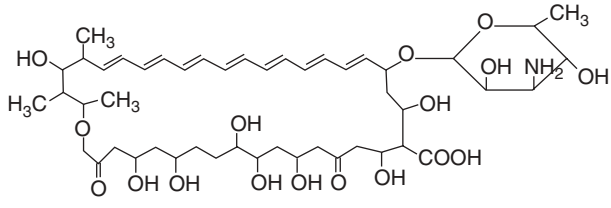
The discovery of nystatin (fungicidin) by Rachel Brown and Elizabeth Hazen in the early 1950s has led to the isolation and characterization of numerous antibiotics. Amphotericin B (fungizone), first isolated in the 1957 from *Streptomyces nodosus*, an actinomycete cultured from the soil of the Orinoco Valley in Venezuela, was the first commercially available systemic antifungal drug; so far, about 200 antifungal agents of this class exist. However, problems associated with the stability, solubility, toxicity and absorption of many such compounds reduced the number of polyenes approved for therapeutic use to only a few. In the UK and the USA, only amphotericin B and nystatin (Figure 8.1) are approved for therapeutic use. While nystatin is useful for superficial mycoses, amphotericin B remains the drug of choice for the treatment of invasive and life-threatening mycoses.

8.2.1 General properties of polyenes

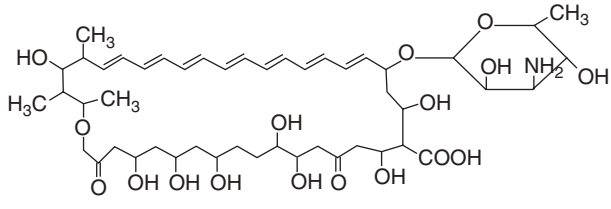
Polyenes are characterized by a large macrolide ring of carbon atoms closed by the formation of an internal ester of lactone (Figure 8.1). The macrolide ring

POLYENES

Amphotericin B



Nystatin



FLUCYTOSINE

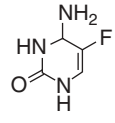


Figure 8.1 Chemical structures of clinically important polyene macrolide and 5-fluorocytosine (4-amino-5-fluoro-2-pyrimidine) antifungals

contains 12–37 carbon atoms; the conjugated double-bond structure is contained exclusively within the cyclic lactone. A number of hydroxyl groups (6–14) are distributed along the macrolide ring on alternate carbon atoms. Amphotericin B has a free carboxyl group and a primary amine group that confer amphoteric properties on the compound, hence the drug's name. Being amphoteric, amphotericin B tends to form channels through the cell membrane causing cell leakage.

The amine group present in some of the polyene antibiotics is associated with an amine sugar connected to the macrolide ring through a glycosidic linkage (Figure 8.1). The carbohydrate moiety in amphotericin B and nystatin is the

mycosamine ($C_6H_{13}O_4N$; 3-amino-3, 6-dideoxymannose) sugar. Polyenes show limited solubility in water and non-polar organic solvents but they dissolve easily in polar organic solvents such as dimethyl sulphoxide or dimethyl formamide.

Although amphotericin B remains the preferred compound for treating systemic mycoses, problems associated with solubility in water, toxicity and ineffectiveness against mould diseases in immunocompromised patients limit its therapeutic potential. Three lipid formulations of amphotericin B (amphotericin B lipid complex, amphotericin B cholesteryl sulfate and liposomal amphotericin B) have been developed and approved for use in the USA. These drug delivery systems offer several advantages over conventional amphotericin B. The parent drug can be introduced in much higher doses (up to ten-fold) compared with conventional amphotericin B. Clinical data suggest that all three formulations are indicated for patients with systemic mycoses who are either intolerant to conventional amphotericin B or have pre-existing renal dysfunction. However, there is debate regarding their use in immunocompromised patients with serious invasive mould infections (aspergillosis and zygomycoses). Amphotericin B cochleate, a novel lipid-based delivery vehicle formed by the precipitation of a negatively charged lipid and a cation (phosphatidylserine) and calcium has been developed. As cochleate lipid particles promote the absorption of amphotericin B from the gastrointestinal tract, the use of this route for therapeutic purposes can now be considered.

8.2.2 Mechanism of action of polyenes

Polyene antibiotics increase cell membrane permeability, which causes leakage of cellular constituents (amino acids, sugars and other metabolites), leading to cell lysis and death. Inhibition of aerobic and anaerobic respiration observed in cells treated with polyenes is thought to be a consequence of leakage of cellular constituents. Polyenes could also cause oxidative damage to the fungal plasmalemma, which may contribute to the fungicidal activity of the drugs. Inhibition of fungal growth by polyenes depends, to a large extent, on the binding of the drug to the cell; only cells that bind appreciable amounts of the drug are sensitive.

Polyene antifungals selectively bind to membrane sterols – ergosterol in fungal cells and cholesterol in mammalian cells. All organisms susceptible to polyenes (yeast, algae, protozoa and mammalian cells) contain sterols in the outer membrane, while those resistant do not. Sensitive fungi can be protected from the inhibitory effects of polyenes by adding sterols to the growth medium, which compete with membrane sterol for the drug.

The interaction of amphotericin B with fungal membrane sterols results in the production of aqueous pores, consisting of an annulus of eight amphotericin B molecules linked hydrophobically to membrane sterols (Figure 8.2). This leads

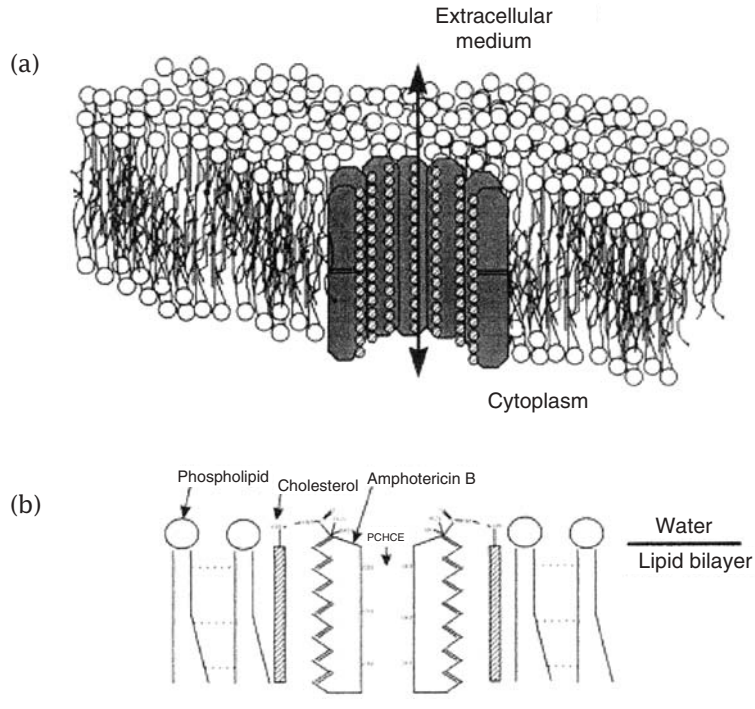


Figure 8.2 Schematic representation of the interaction between amphotericin B and cholesterol in a phospholipid bilayer (reproduced with permission from Ghannoum, M. A. and Rice, L. B. *Clinical Microbiol. Rev.* **12** (4): 501–517, 1999, © 1999, American Society for Microbiology)

to the formation of pores in which the hydroxyl residues of the polyene face inwards to give an effective pore diameter of 0.4–1.0 nm. Leakage of vital cytoplasmic components and death of the cell follows. Figure 8.2(a) shows the conducting pore that forms by the end-to-end union of two wells or half pores. Figure 8.2(b) shows the molecular orientation in an amphotericin B–cholesterol pore. Dotted lines between the hydrocarbon chains of phospholipids represent short-range London–van der Waals forces. Dashed lines represent hydrogen bonds formed between amphotericin B and cholesterol molecules.

The selective mode of action of polyenes is also related to the differential affinity of different polyenes to membrane sterols on target cells. Amphotericin B binds with high affinity to ergosterol in fungal cell membrane. It also binds with low affinity to cholesterol in mammalian cell membrane. Polyene toxicity may also be dependent on the fatty acyl composition of the membrane phospholipids. Changes in the ratio of the various phospholipids may affect the internal viscosity and molecular motion of lipids within the membrane, resulting in variations in sensitivity of different cell types to amphotericin B.

8.2.3 Spectrum of activity of polyenes

Although ineffective against bacteria, a wide variety of fungi and some protozoa are variably susceptible to polyenes. Significant activity against most *Candida* species, *Cryptococcus neoformans*, dimorphic fungi such as *Sporothrix schenkii* and some of the dematiaceous fungi is well established. Many polyenes are toxic to some protozoa of medical importance; *Trichomonas vaginalis*, *Entamoeba* species, *Naegleria* species and *Leishmania donovani* are susceptible to several polyene drugs. Toxicity in this case is caused by loss of intracellular constituents as a result of polyene-induced alterations in membrane permeability. The combination of amphotericin B and flucytosine has additive or synergistic effects against different target cell types. Clear synergistic effects between amphotericin B and flucytosine occur in mice infected with *Cryptococcus neoformans*, *C. albicans*, *Aspergillus* and histoplasmosis.

8.2.4 Pharmacokinetics of polyenes

Since its discovery, amphotericin B has been the gold standard for treating invasive mycoses. In adults, an intravenous (IV) infusion of 0.6 mg/kg yields a peak serum concentration of 1–3 mg/l, which rapidly falls to achieve a prolonged plateau phase of 0.2–0.5 mg/l. Amphotericin B is found primarily in the liver and the spleen; lesser amounts are found in the kidneys and the lungs. These tissue reservoirs elute the drug back into the blood as plasma levels of the drug fall. Amphotericin B follows a biphasic pattern of elimination from serum: an initial half-life of 24–48 hours followed by a long elimination of up to 15 days. This is mainly due to the slow release of the drug from tissues. Detectable levels of the drug can stay in bile for up to 12 days and in urine for up to 35 days following administration. Amphotericin B can remain in the liver and kidneys for as long as 12 months following cessation of therapy. At therapeutic doses, two to five per cent of amphotericin B is excreted in urine and bile.

The pharmacokinetic properties of liposomal forms of amphotericin B significantly differ from those of the conventional micellar form. They are selectively taken up into the reticuloendothelial system and concentrated in the liver, spleen and lungs. Lipid-rich particles are also ingested by phagocytic monocytes; this helps in targeting the drug to sites of infection or inflammation. Lipid formulations of amphotericin B have significantly less nephrotoxicity than deoxycholate amphotericin B, even at higher doses.

8.2.5 Resistance to polyenes

Despite four decades of clinical use, resistance to nystatin and amphotericin B remains rare. Although yeast is intrinsically capable of giving rise to polyene-resistant variants, neither primary nor acquired resistance develops. Polyene-

resistant variants of several *Candida* species are known to exist; all resistant isolates have low membrane ergosterol content. Mutant strains of *Aspergillus fennellial* that are resistant to polyenes have sterols other than ergosterol in the cell membrane. Nystatin-resistant strains of *Saccharomyces cerevisiae* have 5, 6-dihydroergosterol instead of ergosterol as the main membrane sterol component. Resistant organisms with altered sterol content bind smaller amounts of polyenes compared with susceptible ones. Decreased binding capacity of polyenes to mutant strains of *C. albicans* could be attributed to one of several mechanisms: (a) decreased total ergosterol content of the cell without concomitant changes in sterol composition; (b) the presence of low-affinity polyene-binding sterols instead of ergosterol (3-hydroxysterol or 3-oxosterol); (c) reorientation or masking of existing ergosterols so that binding with polyenes is less favoured. The majority of polyene-resistant *Candida* isolates belong to the less common species of *Candida* (*C. tropicalis*, *C. lusitaniae*, *C. glabrata* and *C. guilliermondii*).

8.3 The azole antifungal agents

This group of synthetic antifungal drugs is perhaps the most rapidly expanding group of antifungals. The inhibition of fungal growth by azole derivatives was described in the 1940s and the fungicidal properties of N-substituted imidazoles were described in the 1960s. Clotrimazole and miconazole have proven very important in combating human fungal infections. More than 40 of the β-substituted 1-phenethylimidazole derivatives are known to be potent against fungi, dermatophytes and Gram-positive bacteria. Imidazoles and triazoles are available for treatment of systemic fungal infections. Imidazoles are five-membered ring structures containing two nitrogen atoms with a complex side-chain attached to one of the nitrogen atoms. The structure of triazoles is similar but they contain three nitrogen atoms in the rings (Figure 8.3). Imidazoles in current clinical use are clotrimazole, miconazole, econazole and ketoconazole. Triazole compounds approved for clinical use are itraconazole, fluconazole, voriconazole, lanconazole, ravuconazole and posaconazole.

8.3.1 General properties of the azoles

Imidazoles are soluble in most organic solvents; clotrimazole and itraconazole are insoluble in water. Stock solutions of the drug are stable for up to 1 year at 4 °C in the dark. The ring structure in both imidazoles and triazoles contains a short aliphatic chain in which the second carbon is linked to a halogenated phenyl group; otherwise, considerable variability exists in structure between the two groups (Figure 8.3). The unsubstituted imidazole and the N–C covalent linkage between the imidazole and the rest of the molecule are two features required for antifungal activity.

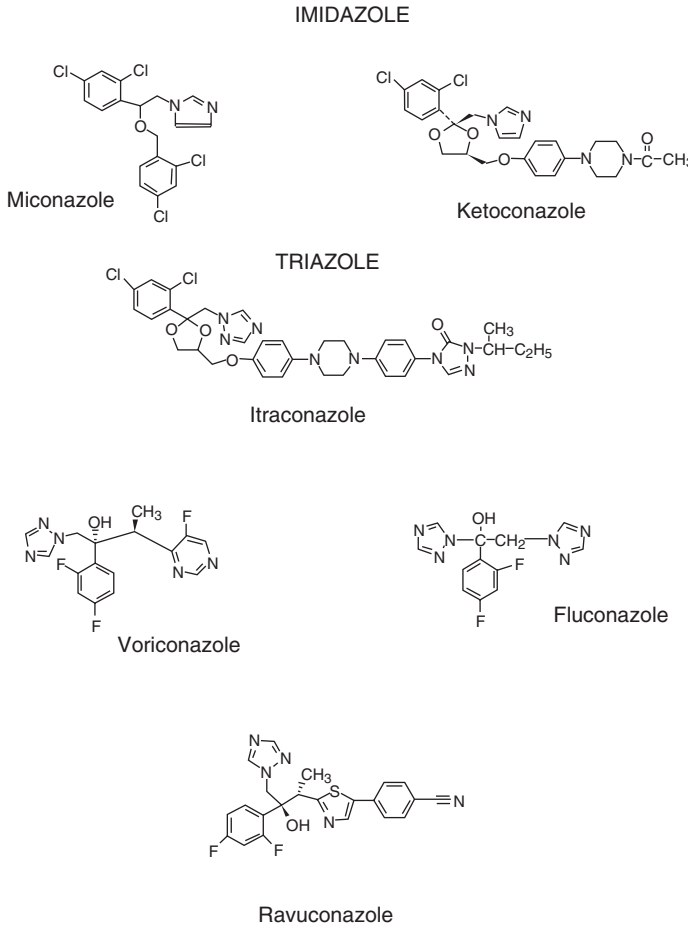


Figure 8.3 Chemical structures of imidazole and triazole antifungal agents

8.3.2 Mechanism of action of azoles

Antifungal activity of azoles is mediated mainly through the inhibition of a cytochrome P450-dependent enzyme involved in the synthesis of ergosterol. In eukaryotic cells, these are integral components of the smooth endoplasmic reticulum and the inner mitochondrial membrane. They contain an iron protoporphyrin moiety located at the active site and play a key role in metabolic and detoxification reactions. Azoles activity is also manifested in inhibiting cytochrome C oxidative and peroxidative enzymes, influencing cell membrane fatty acids causing leakage of proteins and amino acids, inhibiting catalase systems, decreasing fungal adherence and inhibiting germ tube and mycelia formation in *C. albicans*.

The principal molecular target of azoles (fluconazole, itraconazole and voriconazole) is a cytochrome P450-Erg 11P or Cyp 51P according to gene-

based nomenclature. Cytochrome P450-Erg 11P catalyses the oxidative removal of the 14 α-methyl group in lanosterol and/or eburicol by P450 monooxygenase activity. As cytochrome P450-Erg 11P contains an iron protoporphyrin moiety located at the active site, the drug binds to the iron atom via a nitrogen atom in the imidazole or triazole ring. Inhibition of 14 α-demethylase leads to depletion of ergosterol and accumulation of sterol precursors including 14 α-methylated sterol, as shown in Figure 8.4. With ergosterol depleted and replaced by unusual sterols, permeability and fluidity of the fungal cell membrane is altered.

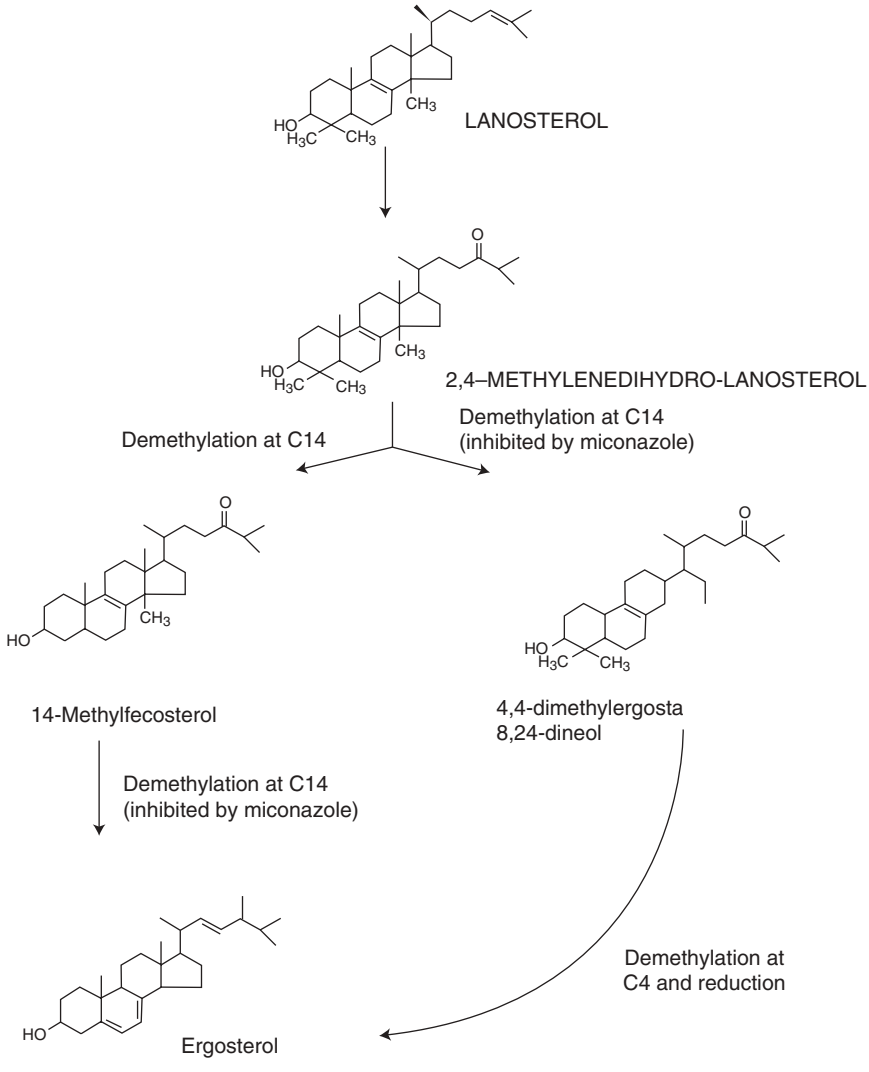


Figure 8.4 Inhibition of sterol demethylation by miconazole

Fluconazole and itraconazole can affect the reduction of obtusifolione to obtusifoliol, which results in the accumulation of methylated sterol precursors (Figure 8.5). Miconazole and ketoconazole can inhibit the ATPase system in the cell membrane of *C. albicans* and other yeasts, which may account for the rapid collapse of the electrochemical gradient and the fall in intracellular ATP. Additionally, at growth inhibitory concentrations, miconazoles and ketoconazoles tend to inhibit the activity of *C. albicans* plasma membrane glucan synthase, chitin synthase, adenylcyclase and 5-nucleotidase enzymes.

Incubation of *C. albicans* and other yeasts at fungistatic concentrations with clotrimazole, miconazole, econazole, voriconazole, posaconazole or ketoconazole results in extensive changes in the cell envelope, especially the plasma membrane – for example, the appearance of holes in the nuclear membrane. At fungicidal concentrations, however, changes in the membrane are more pronounced and include the disappearance of mitochondrial internal structures and the complete loss of the nuclear membrane. Ketoconazole can affect the transformation of *C. albicans* from the budding form to the pseudomycelial form, the prevailing type found in infected individuals.

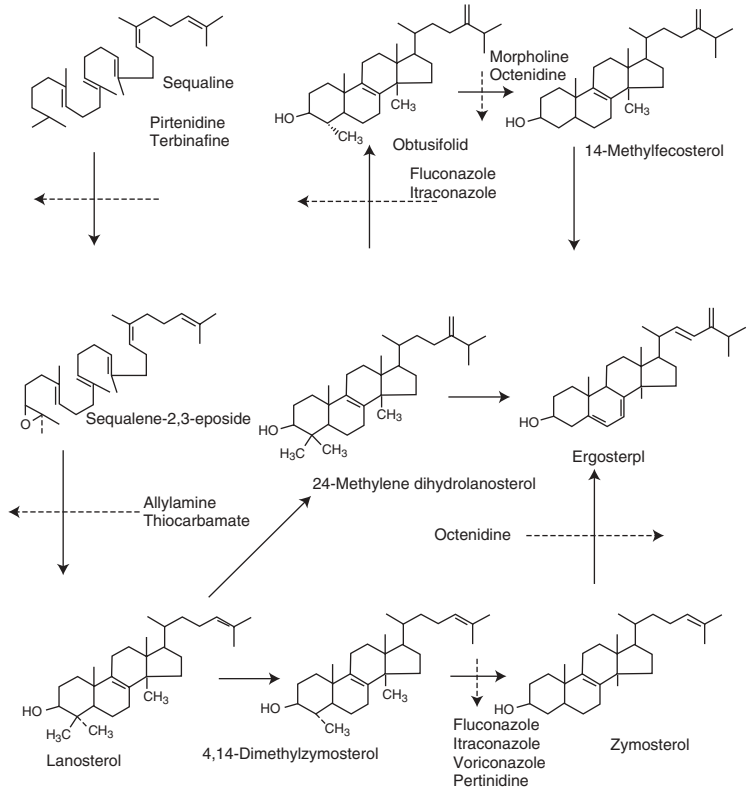


Figure 8.5 Ergosterol biosynthetic pathways and inhibition sites of clinically important antifungal agents

8.3.3 Spectrum of activity of azoles

Azoles possess broad-spectrum antifungal activity in experimental animals and in patients with candidiasis, cryptococcosis, coccidiodomycosis, blastomycosis, and histoplasmosis. In experimental animals with aspergillosis and sporotrichosis, a partial chemotherapeutic response can be observed in the case of itraconazole. Voriconazole and fluconazole can collaborate with human neutrophils and monocytes to enhance the killing of *Aspergillus fumigatus* and *C. albicans*. The drug is also active against *Fusarium* and *Scedosporium* infections and clinical isolates of filamentous species. Gram-positive cocci and bacilli of clinical interest (*Staphylococcus aureus*, *Streptococcus pyogenes*) show marked sensitivity to various azole derivatives with an MIC range of 0.78–100 mg/l.

8.3.4 Pharmacokinetics of azoles

Azoles currently in use differ substantially in their pharmacokinetic properties. Clotrimazole, one of the first azole agents considered for systemic use, is unstable to sustain adequate blood concentrations. Miconazole, the first azole approved for treatment of systemic fungal infections, is administered intravenously (IV) to establish adequate blood concentrations. This leads to local and systemic toxicity with the drug in addition to the restricted ability to reach the CSF, urine and joints.

Ketoconazole produces adequate serum concentrations following oral administration, but its absorption depends on gastric acidity as it must convert to the hydrochloride salt. Fluconazole is available in oral and intravenous preparations. It is effective in children but appropriate mg/kg dosage adjustments must be made depending on the status of the patient. The drug is rapidly and completely absorbed; therefore, serum concentrations using the oral route are comparable to those of the IV route. Gastrointestinal absorption is not greatly affected by gastric acidity, nor does the presence of food substantially hinder absorption. It is minimally metabolized in the liver and excreted largely unchanged in the urine.

Itraconazole is highly lipophilic and almost insoluble in water and diluted acids. It is only ionized at low pH (gastric juice); high concentrations can be achieved in polar organic solvents, cyclodextrins and acidified polyethylene glycol. The average bioavailability of itraconazole after a single oral dose is about 55 per cent, depending on whether the drug is administrated in capsule or solution form. Absorption of the drug is dependent on stomach acidity; it is enhanced by the presence of food in the stomach but reduced in the presence of antacids. Itraconazole plasma concentration is quite low; tissue concentration in general is two to three times greater but in adipose tissue it may go up to 20 times that of plasma levels. The drug persists in tissues for long periods; it is highly metabolized in the liver, with 54 per cent of it removed in faces and

35 per cent in urine. The pharmacokinetics of itraconazole is not affected by renal impairment and the drug is not removed by haemodialysis. Drug metabolism is somewhat reduced in patients with hepatic impairment.

8.3.5 Adverse effects of azoles

Gastrointestinal, hepatic, endocrinologic, metabolic and haematologic toxicities may be associated with the use of azoles (Table 8.1). At conventional doses, azoles are well tolerated even when administered for prolonged periods of time. However, nausea and vomiting may occur in up to 10 per cent of patients receiving ketoconazole at conventional doses. Other adverse effects include headache, fever, fatigue, abdominal pain, diarrhoea, non-fatal uriticaria, exfoliative dermatitis and anaphylaxis.

Transient reversible non-fatal elevations of hepatic transaminase and alkaline phosphate enzymes during the first 2 weeks occasionally occur in 10 per cent of patients receiving ketoconazole. Transient abnormalities of liver function have been observed in three per cent of patients receiving fluconazole. Azoles may interfere with the conversion of lanosterol to cholesterol; the drug blocks 14 α-demethylase, 11 β-hydroxylase and C17, 20-desmolase enzymes. Endocrinologic toxicity is associated with ketoconazole in a dose- and time-dependent

Table 8.1 Tolerability and drug interaction profile of antifungal agents

Drug	Adverse effect
Polyenes	
Amphotericin B	Nephrotoxicity; fever, chills; phlebitis; anaemia;
(deoxycholate)	Gl disturbance
(lipid formulation)	Reduced azotaemia
Fluorinated pyrimidines	
Flucytosine	Bone marrow suppression; hepatotoxicity; Gl disturbance
Azoles	
Saperconazole	Gl disturbance; rare hepatotoxicity
Imidazoles	
Miconazole	Headache; pruritus; thrombophlebitis; hepatotoxicity; autoinduction of hepatic degrading enzymes
Ketoconazole	Gl disturbance; hepatotoxicity
Triazoles	
Itraconazole	GI disturbance; rare hepatotoxicity
Fluconazole	Gl disturbance; rare hepatotoxicity; rare Stevens–Johnson syndrome

manner. Serum testosterone levels are reduced and may result in decreased libido and potency.

At doses greater than 600 mg/d, itraconazole causes endocrinologic toxicity, presumably due to the accumulation of steroid precursors with aldosterone-like effects. These adverse effects are manifested as hypokalaemia and hypertension (Table 8.1).

8.3.6 Resistance to azoles

Although only some of the medically important fungi are inhibited by azoles, the reasonably good safety profile of azoles and triazoles (e.g. fluconazole) is the reason behind the extensive use of these drugs. It is well accepted that imidazoles and triazoles have many of the characteristics required of ideal topical and systemic antifungal drugs. They are active against a wide spectrum of pathogens, they are fungicidal and resistant strains are rare.

Strains of *C. albicans* resistant to azoles are highly unusual; they appear in clinical practice only with protracted exposure to the drug. The mechanism of resistance may involve reduced permeability to azoles and possible alterations in the cell membrane rather than perturbations in cytoplasmic enzymes (Table 8.2).

Table 8.2 Biochemical basis of azole resistance

Mechanism	Cause	Comments
Alteration in drug target (14α-demethylase)	Mutations which alter drug binding but not binding of the endogenous substrate	Target is active (i.e. can catalyse demethylation) but has a reduced affinity towards azoles
Alteration in sterol biosynthesis	Lesions in the $\Delta^{5(6)}$ – desaturase	Results in accumulation of 14α-methyl fecosterol instead of ergosterol
Reduction in the intercellular concentration of target enzyme	Change in membrane lipid and sterols; over-expression of specific drug efflux pumps (CDR1, PDR5 and BEN^r)	Poor penetration across the fungal membrane; active drug efflux
Over-expression of antifungal drug target	Increased copy number of the target enzyme	Results in increased ergosterol synthesis; contributes to cross-resistance between fluconazole and itraconazole

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Pathogenic strains resistant to both polyenes and azoles have, in general, higher lipid content and lower ratios of polar to neutral membrane lipids compared with sensitive strains or strains resistant only to azoles. Altered membrane sterol patterns may provide a common basis for the dual resistance by preventing polyene binding and reducing permeability to azoles.

8.4 Flucytosine

Flucytosine or 5-fluorocytosine (5-FC) is a synthetic fluorinated pyrimidine used as an oral antimycotic agent. It was first synthesized in the 1950s as a spin-off of work in cytostatic and antineoplastic agents. 5-FC lacks such activities but it has noticeable antifungal activity. Currently, 5-FC is used as an adjunct to amphotericin B therapy because amphotericin B increases the uptake of 5-FC by increasing fungal cell membrane permeability. The activity of 5-FC is enhanced when used in combination with fluconazole against *C. neoformans* and *C. albicans*.

8.4.1 Basic properties of flucytosine

The chemical formula of 5-FC is 4-amino-5-fluoro-2-pyrimidine; the compound has a molecular weight of 129 kDa (Figure 8.1). The drug is odourless, white and crystalline, it is relatively stable at normal temperatures and it is soluble in water up to 1.2 per cent. When 5-FC is maintained at low temperatures it tends to crystallize. It tends to partially deaminate to 5-fluorouracil (5-FU) when stored at high temperatures and once taken by cells.

8.4.2 Mechanism of action of flucytosine

The antifungal activity of 5-FC is mediated through one of two mechanisms: (a) disruption of DNA synthesis and/or (b) alteration of the amino-acid pool as shown in Figure 8.6. Initially, 5-FC enters susceptible cells by means of cytosine permease, which is usually responsible for the uptake of cytosine, adenine, guanine and hypoxanthine. Once inside the cell, 5-FC is converted to 5-fluorouracil (5-FU) by cytosine deaminase. Inside target cells, 5-FU is then converted by uridine monophosphate pyrophosphorylase to 5-fluorouridylic acid (FUMP), which is phosphorylated further and incorporated into RNA, resulting in disruption of protein synthesis. Extensive replacement of uracil by 5-FC in fungal RNA can lead to alterations in the amino-acid pool. Some 5-FU can be converted to 5-fluorodeoxyuridine monophosphate, which functions as a potent inhibitor of thymidylate synthase, one of the enzymes involved in DNA

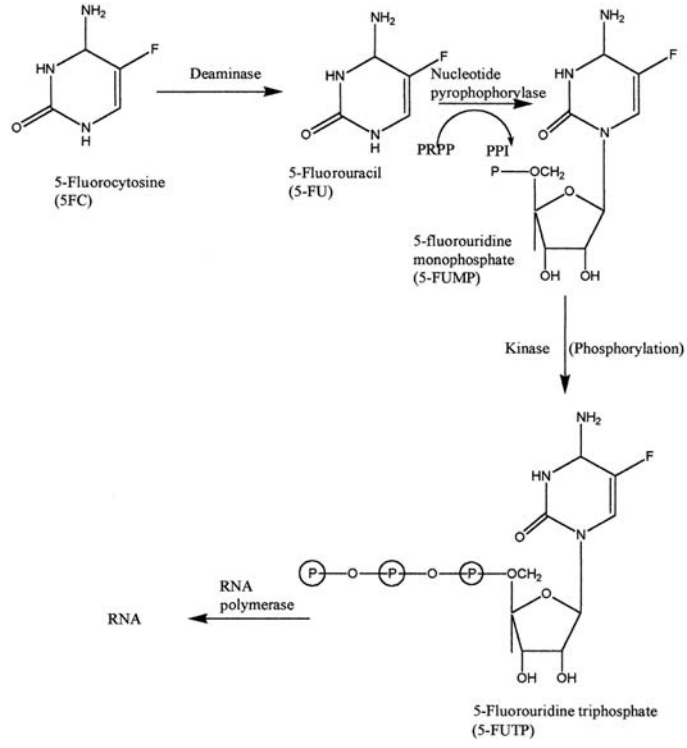


Figure 8.6 Metabolic conversions and mode of action of 5-FC

synthesis and nuclear division. Inhibition of DNA synthesis in *C. albicans* can take place before 5-FU incorporation into RNA or inhibition of protein synthesis. Resistant strains of *C. neoformans* incorporate 5-FC into RNA at levels comparable with sensitive strains. This could mean that resistance inhibition of DNA synthesis is more important than the production of aberrant RNA in mediating the effects of 5-FC. The drug incorporates in large quantities into the 80S ribosomal subunits in *C. albicans*. The number of ribosomes synthesized in the presence of high concentrations of 5-FC is greatly reduced (Figure 8.6).

Morphological and ultrastructural changes that occur in *C. albicans* cells include increased cell diameter if growing at sub-inhibitory concentrations of 5-FC. This results mainly from continued or excessive synthesis of carbohydrates and proteins. Following a 12 hour incubation period, the nucleus is enlarged and become translucent with filamentous components; the cell wall becomes progressively thinner. *C. albicans* and *C. neoformans* show increased budding. Most of these changes can be attributed to unbalanced growth activities, as treated cells are incapable of synthesizing DNA while retaining residual metabolic potential.

8.4.3 Spectrum of 5-flucytosine antifungal activity

5-FC displays significant antifungal activity against *Candida*, *Torulopsis*, *Cryptococcus* and *Geotrichum* genera. It also exhibits moderate activity against *Aspergillus* species and chromomycosis-causing dematiaceous fungi. Other fungi of medical importance such as *Coccidioides*, *Histoplasma* and dermatophytes do not respond to 5-FC treatment. 5-FC exerts both fungicidal and fungistatic activity against *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata and C. neoformans*. The fungicidal effects usually occur at relatively high concentration and after prolonged exposure.

8.4.4 Pharmacokinetics of 5-flucytosine

Upon oral intake, 5-FC is completely absorbed from the intestine and therefore exhibits good bioavailability properties. About 80 per cent of the drug is excreted unchanged in the urine; negligible quantities bind to serum proteins. Administration of 150 mg/kg/day results in peak serum concentrations of 50–80 mg/l within 1–2 hours in adults with normal renal function. The drug accumulates in patients with azotaemia; this could result in toxic effects unless the dosage is reduced. CSF concentration can exceed 70 per cent of that of serum concentration, making the drug suitable to treat mycoses of the central nervous system. 5-FC (ancobon) is available in 250 mg and 500 mg capsules. The initial dosage in patients with normal renal function is 37.5 mg/kg every 6 hours (150 mg/kg/day).

8.4.5 Adverse effects of 5-flucytosine

Gastrointestinal-tract-related symptoms such as diarrhoea, nausea and vomiting are among the most common but least serious effects associated with 5-FC therapy. Hepatitis can occur at concentrations of 50 μg/ml or higher; it usually resolves in several days to a few weeks. Bone marrow suppression in the form of neutropenia, thrombocytopenia and pancytopenia may develop following treatment.

Human cells do not deaminate flucytosine; metabolites of 5-fluorouracil can be found in the urine and 5-fluorouracil can be found in blood at low concentrations. It is possible that colonic bacteria such as *Escherichia coli* that contain cytosine deaminase could deaminate flucytosine, which is reabsorbed. This may explain why flucytosine enteritis is largely confined to the colon. Variations in the potential of different colonic bacteria to deaminate the drug may explain why some patients can maintain flucytosine blood levels at 150 μg/ml for 6 weeks without toxicity.

8.4.6 Resistance to flucytosine

Many fungi are either inherently resistant to 5-FC or they develop resistance to the drug. Resistance develops by loss of cytosine permease, loss of cytosine deaminase, deficiency in uridine monophosphate pyrophosphorylase or increased *de novo* synthesis of pyrimidine via increased orotidylic acid pyrophosphorylase and orotidylic decarboxylase. Loss of feedback regulation of aspartic transcarbamylase by ATP leading to increased *de novo* synthesis of pyrimidine may also be one the resistance mechanisms to 5-FC. Decreased uptake of 5-FC evident in *S. cerevisiae* and *C. glabrata* is not the major mechanism of resistance in *C. albicans* or in *C. neoformans*. The frequency of *C. albicans* resistance to 5-FC at concentrations higher than 25 μg/ml is generally greater among strains of serotype B than serotype A. Serotype B, though it comprises a small minority in most clinical isolate collections, is usually responsible for the majority of primary resistance instances.

8.4.7 Use of 5-flucytosine in combination with amphotericin B

5-FC is not administered alone out of concerns for development of secondary drug resistance, which can be profound and accompanied by clinical deterioration. The combination of 5-FC and amphotericin B has proved useful in rapidly clearing CSF when used to treat cryptococcal meningitis in non-HIV-infected patients. It has also proved effective against large cryptococcal intracerebral masses, minimizing the need for surgical intervention. The combination of 5-FC with amphotericin B is therefore recommended for treating central nervous system (CNS) cryptococcosis and candidiasis, *Candida* endophthalmitis, renal and hepatosplenic candidiasis, *Candida* thrombophlebitis of the great veins, aspergillosis and CNS phaeohyphomycosis.

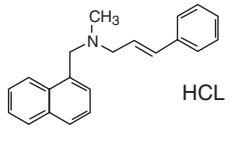
8.5 Novel antifungal agents

The need to develop new classes of antifungal agents never ceases to expand. It stems primarily from the continuous rise in the incidence of fungal infections and the limited number of effective side-effect-free antifungals. The development of resistance to antifungal drugs, currently appreciated as an emerging problem, makes the search for newer agents critical. A number of new antifungal agents including allylamines and thiocarbamates, octenidine and pirtenidine, morpholines and histatins have been developed and approved, or are in the process of being approved for clinical use.

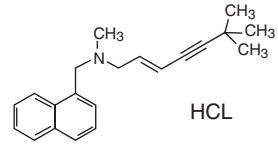
8.5.1 Allylamines and thiocarbamates

Naftifine and terbinafine are the two major allylamines in clinical use and tolnaftate is the only thiocarbamate available for use. Naftifine is used as a topical agent while terbinafine is administered orally. These are two synthetic compounds with a chemical structure similar to naphthalene ring substituted at the 1-position with an aliphatic chain (Figure 8.7). Both allylamines and thiocarbamates function as non-competitive inhibitors of squalene epoxidase, an enzyme involved in the conversion of squalene to lanosterol, which is an essential step in the synthesis of the fungal cell membrane.

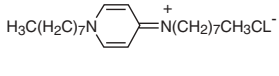
Cell death is dependent on the accumulation of squalene rather than ergosterol deficiency, as high levels of squalene increase membrane permeability, leading to disruption of cellular organization. Terbinafine inhibits the growth of dermatophytic fungi *in vitro* at concentrations of 0.01 μg/ml. Naftifine hydrochloride as one per cent cream is effective against tinea cruris and tinea corporis but *C. albicans* is much less susceptible to naftifine. Resistance to allylamines and thiocarbamates is yet to be reported for medically important fungi. However, allylamine-resistant strains of *S. cerevisiae* and the plant pathogen *Ustilago maydis* are known.



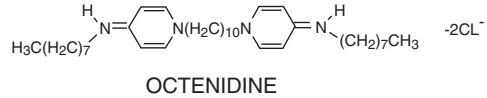
Naftifine



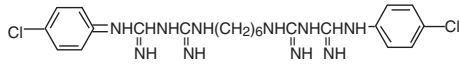
Terbinafine



PIRTENIDINE



OCTENIDINE



CHLORHEXIDINE

Figure 8.7 Chemical structures of allylamines (naftifine and terbinafine), pirtenidine, octenidine and chlorhexidine

8.5.2 Octenidine and pirtenidine

Octenidine and pirtenidine, which are structurally similar to chlorhexidine (Figure 8.7), have been developed for use as antibacterial mouthwashes. They are both alkylpyridinylidine–octanamine derivatives; they cause extensive leakage of cytoplasmic contents from *C. albicans* and *S. cerevisiae*. These changes are correlated with gross morphological and ultrastructural changes in treated cells. Treatment of *C. albicans* with subinhibitory concentrations of octenidine or pirtenidine alters membrane lipid and sterol contents. A significant increase in squalene and 4, 14-dimethylzymosterol occurs in pirtenidine-treated cells. Octenidine-treated cells show increased zymosterol and obtusifoliol contents; these changes affect ergosterol biosynthesis.

8.5.3 Morpholines

Morpholines are synthetic phenylmorpholine derivatives with amorolfine as the sole representative in clinical use. The drug, discovered in the 1980s, shows a broad range of MICs for *Candida* isolates *in vitro*. It acts on the ergosterol pathway, inhibiting the Erg24P (Δ14-reductase) reaction and the Erg2P (Δ8–Δ7 isomerase enzyme) reaction. Amorolfine is used only for topical treatment of superficial mycoses; resistance to the drug is yet to be documented.

8.5.4 Histatins

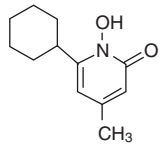
Histatins represent a family of small, cationic, histidine-rich peptides secreted in the saliva by human parotid and submandibular–sublingual glands. Histatins consist of 12 members, the three major ones being 1, 3 and 5, which contain 38, 32 and 24 amino acids respectively. These participate in various biological processes such as the formation of the enamel pellicle of the teeth, induction of histamine release, inhibition of haemagglutination, inhibition of protease activity and neutralization of lipopolysaccharide. They exhibit fungicidal activity against several *Candida* species (*C. albicans*, *C. glabrata* and *C. krusei*), *S. cerevisiae*, *C. neoformans* and *A. fumigatus*. They also show modest bactericidal or inhibitory effects on *Streptococcus mutans*, the bacterium responsible for dental carries, *Streptococcus mitis*, *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*.

Histatins kill or inhibit *Candida* species at physiological concentrations (15–30 μM). The action of histatins on the surface of target cells may differ from that of larger antimicrobial peptides. The 14-amino-acid peptide corresponding to the C-terminal at histatin 5 and the middle of histatin 3 as well as the α-helical conformation typical of many histatins are the major structural requirements for eliciting appreciable candidacidal activity. In close proximity to the

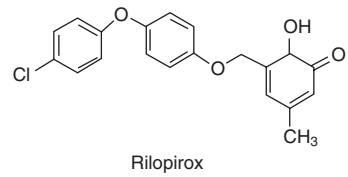
plasma membrane, histatins may change from the random coiling conformation in aqueous solution into an ordered α-helical one. Histatins bind to the *C. albicans* cell membrane but not to mammalian cell membrane, hence the selective candidacidal activity of these proteins. Histatin 5 targets the mitochondria and results in loss of transmembrane potential. Inhibition of mitochondrial ATP synthesis protects *C. albicans* against the fungicidal activity of histatin 5 mainly due to reduced accumulation of the peptide.

8.6 Miscellaneous antifungal agents

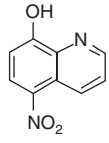
Significant antifungal activity of the quinoline nitroxoline (Figure 8.8), a urinary antiseptic, is known to exist for a considerable number of pathogenic *Candida* species. The MIC of nitroxoline is in the range of 0.25–2 μg/mL for represen-



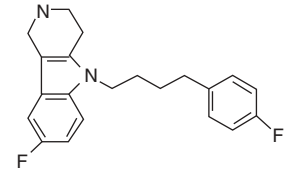
Ciclopirox



Rilopirox



5,nitro-8-hydroxyquinoline (Nitroxoline)



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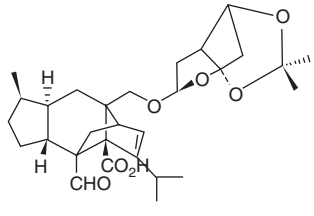
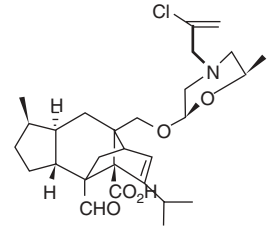


SORDARINE (GM193663)



SORDARINE (GM531920)

Figure 8.8 Chemical structure of two sordarins (GM 193663 and GM 531920) in addition to some promising antifungal compounds currently under development

tative strains of *Candida*. The drug appears to be superior to miconazole and ketoconazole. Compound A-39806 (Figure 8.8) shows antifungal activity against several *Candida* species, *C. albidus* and *A. niger*. These non-azole compounds increase the 4, 14-dimethyl sterols and concurrently decrease desmethyl sterols indicative of lanosterol 14-α-demethylase inhibition.

Ciclopirox olamine, *ciclopirox* and *rilopirox* (Figure 8.8) are hydroxypyridones with *in vitro* activity against a broad spectrum of medically important fungi including dermatophytes, yeasts and moulds. They also exhibit *in vitro* activity against a number of Gram-positive and Gram-negative bacteria. These compounds are not related to azoles or any other antifungal agents. Hydroxypyridones do not affect sterol synthesis of fungal pathogens. Their main antifungal mechanism centres on the inhibition of cellular uptake of essential compounds; at high concentrations, they can alter membrane permeability.

Sordarins merit mention owing to their unique mechanisms of action on fungi. They were discovered by routine screening, abandoned in the early 1970s and revisited later as a result of prospective screening for inhibitors of *C. albicans* protein synthesis *in vitro*. Sordarins inhibit protein synthesis by blocking the function of fungal translation elongation factor 2 (EF2). It is worth noting that *C. albicans* EF2 displays more than 85 per cent amino-acid sequence homology to the human equivalent, making the likelihood to toxic side-effects to host cells relatively high. However, their high specificity for respective fungal targets and the relative ease with which new variants are synthesized holds promise for positive future developments in this area.

Pradimicin A (BMY-28567) and *pradimicin B* (BMY-28864), described in the late 1980s, and *pradimicin B analogue* (BMS-181184), a recently described member, all represent a new single class of antifungal compounds. BMS-181184, a water-soluble derivative of pradimicin, has an MIC between 1 and 8 μg/ml against the majority of isolates of *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. lusitaniae* and *C. neoformans* that have been tested. The definitive mode of action of pradimicin remains unclear, but appears to involve initial calcium-dependent complexing of the free carboxyl group with the saccharide portion of cell surface mannoproteins (Table 8.3). The agent then acts on the membrane, causing leakage of intracellular potassium.

The fungal cell wall contains compounds, such as mannan, chitin and glucans, which are unique to the kingdom *Fungi*. A number of compounds that have the ability to affect the cell wall of fungi have been described over the past 30 years. Three groups of compounds (aculeacins, echinocandins and papulacandins) are specific inhibitors of fungal β (1,3)-glucan synthase. *Echinocandins* are actively pursued in clinical trials to evaluate their safety, tolerability and efficacy against candidiasis. Discovered by random screening in the 1970s, echinocandins are fungal secondary metabolites comprising a cyclic hexapeptide core with a lipid side chain responsible for antifungal activity. A modified form of echinocandin B, cilofungin, was developed to the point of phase 2 trials, but then abandoned due to increased toxicity. In the late 1990s, three echinocandin compounds

Table 8.3 New targets for antifungal agents

Target	Compound	Mechanism of action
Fungal cell wall		
1. Chitin	Polyoxins Nikkomycin Demethylallosamidin	Chitin synthase inhibitor; chitinase inhibitor
2. Glucan	Aculeacins Echinocadins Papulacandins	β-(1,3)-glucan synthase inhibitor
3. Mannoprotein	Pradimicin A Benanomycin A	Calcium – dependent complexing with the saccharide of mannoprotein, thereby disrupting the cytoplamic membrane, causing leakage of intracellular potassium
Plasma membrane		
1. Ergosterol synthesis	Octenidine Pirtenidine A-39806	Affect ergosterol biosynthesis by inhibition of 14α-demethylase
2. Sphingolipid	Sphingofungins	Inhibition of serine palmitoyltrasferase
3. Protone ATPase	Floimycin (concanamycin A) Hydroxypyridones	Inhibitor of V-type H^+-ATPase Inhibit ATP synthesis and cellular uptake of essential component
Intermediary metabolism		
1. Amino acids	Cispentacin RI-331	Interfering with amino-acid synthesis Inhibit homoserine dehydrogenase
2. Polyamines	Eflorinithine (difluoromethyl ornithine)	Inhibitor of ornithine decarboxylase

(anidulafungin, caspofungin and micafungin, Figure 8.9) entered clinical development and evaluation.

These compounds act as specific noncompetitive inhibitors of β-(1,3)-glucan synthetase, a large (210 kDa) integral membrane heterodimeric protein. Treatment of fungi with echinocandin inhibits synthesis of the structural glucan component without affecting nucleic acid or mannan synthesis. In *S. cerevisiae*, two proteins (Fks1p and Fks2p) are regulated by a GTP-binding peptide called Rho1p and by elements of the calcineurin pathway. Homologues to the three gene products have been found in *C. albicans*, but it seems that the Fks2p homologue is not expressed in growing cells. Although Fks1p is the component to

ECHINOCANDINS

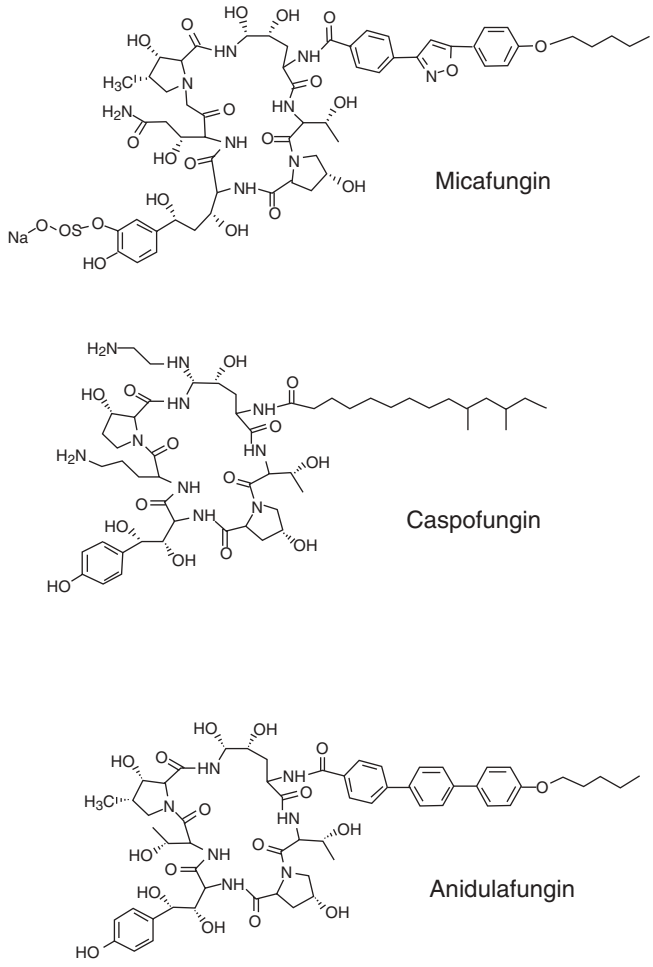


Figure 8.9 Chemical structure of anidulafungin, caspofungin and micafungin

which echinocandins bind, it may not necessarily be the catalytic subunit that mediates the non-competitive inhibitory effects on glucan synthesis.

Besides their inhibitory action, inhibitors of glucan synthesis have secondary effects on other components of intact cells. These include reduction in the ergosterol and lanosterol content as well as increased cell wall chitin content. Inhibition of β-(1,3)-glucan synthetase results in cytological and ultrastructural changes in fungi characterized by growth as pseudohyphae, thickened cell wall and buds failing to separate from mother cells. Cells also become osmotically sensitive, with lysis being restricted largely to the growing tips of budding cells.

Chitin synthesis is inhibited competitively by polyoxin and nikkomycin, nucleoside–peptide antibiotics produced by soil strains of *Streptomycetes*. These agents specifically inhibit chitin synthase by acting as mimics or decoys of the enzyme substrate (uridine diphosphate-N-acetylglucosamine). *In vitro* susceptibility testing of nikkomycins X and Z against various fungi shows moderate susceptibility of *C. albicans* and *C. neoformans* to these compounds. Activity against *C. albicans* and *C. neoformans* improves significantly when nikkomycin Z is used in combination with fluconazole and itraconazole.

8.7 New strategies and future prospects

The applicability, efficacy and therapeutic value of most current antifungals can be compromised by increased toxicity, narrow spectrum of activity, development of resistance and limited range of specific molecular targets in fungal cells. Therefore, the development of new classes of antifungal compounds is continuously needed. Work to develop and introduce new protective and therapeutic strategies that aid or altogether replace chemotherapy is underway.

8.7.1 New antifungals

Should new antifungal agents be sought and developed, they should possess a number of features to better deal with the multitude of problems associated with the use of conventional antifungals. Chief among these features are enhanced potency, broad spectrum of activity, reduced or limited toxicity, flexible mode of administration and favourable pharmacokinetics (bioavailability and effective tissue penetration). New triazole agents in development (posaconazole, ravuconazole and voriconazole) and echinocandins (anidulafungin, caspofungin and micafungin) go some way to meeting these requirements. However, the emergence of resistance to these and other novel antifungals is possible. Therefore, the battle between fungi and man is continuous; identification of new targets and new inhibitors may be the focus of research for many years to come.

8.7.2 Protective and therapeutic antibodies

Despite the limited role of antibody immunity in protection against fungal infections, monoclonal antibodies (mAb) generated against certain *C. neoformans* and *C. albicans* antigens can be protective to naïve mice, depending on the antigen against which the mAb is raised. Accordingly, the identification of immunodominant antigenic moieties to be targeted for the production of monoclonal antibodies is of prime importance. *Candida* secretory aspartic proteinase (Sap) 2, mannoprotein and surface mannan are among the immunodominant

candidal antigens. Intravaginal and intranasal immunization with mannoprotein extracts or secreted *C. albicans* Sap2 can confer protection against vaginal candidosis, especially when mixed with the appropriate adjuvant. For *C. neoformans*, the isotype (or subisotype) of the antibody can also be critical in determining whether the antibody is protective or not.

Protective antibodies can be used as adjuncts of standard chemotherapy. The efficacy of amphotericin B, fluconazole and 5-FC is enhanced when used in conjunction with protective antibodies against *C. neoformans*. The existence of protective antibodies against fungal infections has already opened the door for the development of monoclonal-antibody-based vaccines and those that induce the production of antibody responses.

8.7.3 Antifungal vaccines

Therapeutic vaccines (immunogens) can be used to boost antigen-specific immune responses during active infection. So far, antifungal vaccines that offer the minimum safety and efficacy requirements have yet to be introduced. Although avirulent strains of fungi are known to exist, common vaccination approaches, which rely on the use of an attenuated strain, are not as applicable in the case of fungi as in the case of viruses or bacteria. The features that make a fungus avirulent would have to be stable within the host. In the case of deep fungal infections, the fungus would have to be totally eliminated by the immune system to prevent the formation of a dormant fungal infection that can be reactivated in the future. Attenuated forms of deep-tissue-infecting fungi generated by genetically incapacitating important growth or pathogenic components have proven useful in experimental *H. capsulatum* infections; however, the aforementioned concerns remain valid.

Murine monoclonal antibody (mAb) KT4, a neutralizing antibody of the yeast *Pichia anomala* killer toxin (PaKT), was able to induce the secretion of anti-idiotype antibodies. These anti-idiotypes were shown to have direct *in vitro* killing activity against PaKT-susceptible *C. albicans* cells. They were also able to generate passive protection in unvaccinated animals. The WI-1 gene codes for a protein that enables the fungus to stick and stay in the lungs. The WI-1 protein is among the immunodominant immunogens of *Blastomyces dermatidis*. A WI-1 gene knockout strain of *B. dermatidis* was avirulent in laboratory mice but was able to induce T-cell responses that fully immunized the mice against all strains tested at lethal doses.

8.7.4 Protective oligonucleotides

RNA targets such as ribosomal RNA, messenger RNA and RNase P RNA require folding into secondary and tertiary structures to function properly.

Current work in this area focuses on what is called the oligonucleotide-directed misfolding of RNA (OMMiR), which is a method that uses short oligonucleotides to stabilize improperly folded inactive RNA structures. Two oligonucleotides (TACCTTTC and TCTACGACGGCC) were recently shown to inhibit 50 per cent of group I intron splicing in *C. albicans*. This approach holds some potential as a therapeutic strategy applicable to RNAs with structural folding requirements. The search for potential oligonucleotides and target RNAs in pathogenic agents will no doubt attract the attention of mycologists concerned with the development of effective antifungals.

8.8 Conclusion

Recent years have seen an increase in the incidence of potentially fatal fungal infections due to the increasing use of immunosuppressive therapy during medical therapies and the enhanced ability of medical personnel to extend the lives of patients who, in previous decades, would have died at an earlier stage. Part of the problem in combating such infections has been the rise in the occurrence of strains resistant to conventional antifungal drugs and the toxicity associated with specific compounds used in antifungal therapy. While the search for new and better antifungal drugs continues, the success of this search will be judged not only on the efficacy of a particular drug against one or more fungal pathogen but also on its tolerability within the patient. It is this latter parameter that makes the development of antifungal vaccines such an attractive possibility.

8.9 Further reading

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8.10 Revision Questions

Q 8.1 What does the binding of polyene antifungals to the cytoplasmic membrane of fungi cause?

Q 8.2 What antifungal agent binds preferentially to fungal ergosterol, altering cellular permeability?

Q 8.3 Which polyene macrolide is only used topically, due to systemic toxicity, is active against most *Candida* species and is most commonly used for suppression of local candidal infection?

Q 8.4 What is the drug of choice for nearly all life-threatening mycotic infections and usually used in the initial induction regimen?

Q 8.5 What are the modes of action of flucytosine?

Q 8.6 What is the mode of action of azole antifungal agents such as ketoconazole and itraconazole?

Q 8.7 Which azole is most commonly used for topical treatment of candidiasis?

Q 8.8 Which systemic drugs are used for the treatment of coccidiodomycosis?

Q 8.9 What is the most potent of the currently available antifungal azoles?

Q 8.10 Which antifungal agent inhibits fungal squalene epoxidase?

Q 8.11 Which topical allylamine is used for treating tinea cruris and tinea corporis?

Q 8.12 Which antifungal drug inhibits fungal β-(1,3)-glucan synthetase?

Q 8.13 Which drugs inhibit chitin synthesis?

9
Fungal Pathogens of Plants

Fiona Doohan

9.1 Fungal pathogens of plants

A wide range of fungi cause diseases of plants. To be classified as a fungal plant pathogen or phytopathogen (phyto = plant), a fungus should, if possible, satisfy *Koch's postulates* or rules. Koch, one of the original researchers of tuberculosis in the 19th century, determined that an organism was the cause of an infectious disease if it can (a) be isolated from a diseased host, (b) be cultured in the laboratory, (c) cause the same disease upon re-introduction into another host plant and (d) be re-isolated from that host. However, some fungal pathogens cannot be cultured, or in some cases it is not easy to carry out Koch's tests.

Since early in the 19th century, thousands of fungi have been recognized as parasites of plants and almost all plants are hosts to particular fungal diseases. *Parasitism* occurs where one species lives off another, as distinct from *symbiosis*, where different species live in harmony with each other and the relationship is mutually beneficial, or *saprophytism*, where organisms grow on dead organic matter.

Plant-pathogenic fungi can be classified as follows.

- *Biotrophs*: only grow and multiply when in contact with their host plants (and therefore cannot be cultured on nutrient media), e.g. the fungi that cause rusts, powdery mildews and downy mildews.
- *Non-obligate pathogens*: grow and multiply on dead organic matter (and can therefore be cultured on nutrient media) as well as on living host tissue. These can be further distinguished as facultative saprophytes or facultative

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pathogens. Facultative saprophytes complete most of their life cycle as parasites, but under certain conditions they grow on dead organic matter. Conversely, facultative parasites complete most of their life cycle on dead organic matter but under certain conditions they attack and parasitize living plants.

9.2 Disease symptoms

Plant disease *symptoms* caused by fungal parasitism include the following.

- *Spots:* localized lesions on host leaves.
- *Wilts*: fungal colonization of root or stem vascular tissue and subsequent inhibition of translocation leading to wilting.
- *Blight*: browning of leaves, branches, twigs or floral organs.
- *Rots:* disintegration of roots, stems, fruits, tubers, fleshy leaves, corms or bulbs.
- *Cankers:* localized, often sunken, wounds on woody stems.
- *Die back*: necrosis of twigs initiated at the tip and advancing to the twig base.
- *Abnormal growth*: enlarged gall-like or wartlike swelling of plant stems, roots, tubers, leaves or twigs, root and shoot proliferation, etc.
- *Damping off*: rapid death and collapse of young seedlings.
- *Decline*: loss of plant vigour; retarded development.
- *Anthracnose*: necrotic (often sunken) ulcer-like blemishes on stems, fruits, leaves or flowers.
- *Scab:* localized lesions of scabby appearance on host fruit, leaves, tubers.
- *Rusts:* many small, often rust-coloured lesions on leaves or stems.
- *Mildews*: chlorotic or necrotic leaves, stems and fruits covered with mycelium and fruiting bodies of the fungus, often giving a white 'woolly' appearance.

Many diseases are associated with more than one of these symptoms. Table 9.1 lists some of the economically significant fungal pathogens, associated symptoms and host plants.

Table 9.1 Examples of economically significant fungal diseases, causal organisms, hosts and associated symptoms*

Disease	Fungus	Hosts	Symptoms
Vascular wilts			
Fusarium wilt	*Fusarium oxysporum*	Most vegetable and flower crops, cotton, tobacco, banana, plantain, coffee, turfgrass, ginger, soybean	Wilting, vein clearing in younger leaflets, epinasty, stunting and yellowing of older leaves
Verticillium wilt	*Verticillium dahliae* and *Verticillium albo-atrum*	Many vegetables, flowers, field crops, fruit trees, roses and forest trees	Similar to Fusarium wilt
Dutch elm disease	*Ophiostoma ulmi* and *Ophiostoma novo-ulmi*	Elm	Wilting, yellowing/browning of leaves, brown/green streaks in the infected sapwood underneath the bark of infected branches
Oak wilt	*Ceratocystis fagacearum*	Oaks	Downward wilting and browning of leaves, defoliation. Brown discoloration of the sapwood underneath the bark of infected branches
Blights			
Late blight of potato	*Phytophthora infestans*	Potato and tomato	Water-soaked lesions turning to dead brown blighted areas on lower leaves, white woolly growth on underside of infected leaves; tubers have dark surface blotches and internal watery dark rotted tissue
Downy mildews	Several genera, e.g. *Erysiphe*	Wide range of crops, ornamentals and shrubs	Powdery growth covering shoots, leaf surfaces and sometimes flowers
Alternaria blight (early blight)	*Alternaria solani*	Potato, tomato	Dark lesions on stems, leaves, potato tuber lesions and internal dry rotting, tomato blossom blight, fruit rot and stem lesions
Helminthosporium leaf blight	*Cochliobolus sativus*	Cereals and grasses	Dark brown spotting of leaves (also causes root rot, seedling blight and head blight)

Table 9.1 *Continued*

Diseases	Fungus	Hosts	Symptoms
Botrytis blossom blight (and rots)	*Botrytis* spp. (e.g. *Botrytis cinerea*)	Ornamentals and fruit trees	Water-soaked and rotting blossoms, grey/brown powdery lesions on fruit, leaves, stems and bulbs, rotting of fruit
Fusarium head blight	*Fusarium* spp.	Cereals	Premature bleaching of cereal spikelets and shrivelled pale/pink grain
Rots			
Phytophthora root rots	*Phytophthora* spp.	Fruit, forest, ornamental trees and shrubs, annual vegetables and ornamentals	Rotting of roots, plant stunting and wilting, death in severe cases
Damping off and associated rots	Several genera, e.g. *Pythium* spp.	Many hosts; broad-leaved weeds and grasses are very susceptible	Seedling death, rotting of seed, roots, and fruit in contact with soil
Soft rot	*Rhizopus* spp.	Fruit and vegetables	Softening and rotting of soft fleshy organs and fruit
Brown rot of stone fruits	*Monilia* spp.	Stone fruits	Brown rotting of stone fruits
Anthracnose	Several genera, e.g. *Colletotrichum* spp.	Fruits, fruit and some forest trees, beans, cotton, ornamentals, rye, etc.	Dark sunken lesions on stems or fruits, may cause a rot of fruit
Leaf and stem spots			
Septoria leaf spot	*Mycosphaerella graminicola*	Cereals (primarily wheat)	Grey to brown water-soaked leaf lesions that, when mature, bear black visible pycnidia
Scabs			
Apple scab	*Venturia inaequalis*	Apples	Dark lesions on fruit, leaves, and sometimes on stems and bud scales

Rusts			
Black stem rust of cereals	*Puccinia graminis*	Cereals	Diamond-shaped raised orange/red powdery lesions on leaves, stems, cereal heads, that when mature, bear black teliospores
Coffee rust	*Hemileia vastatrix*	Coffee	Yellow/orange oval lesions on leaves with powdery orange/yellow lesions on the leaf undersurface, infected leaves eventually drop off
Smuts			
Covered smut of oats	*Ustilago hordei*	Oats	Grain appears black due to its replacement with black fungal spore masses
Covered smut (bunt) of wheat	*Tilletia* spp.	Wheat	As above
Powdery mildews			
Powdery mildew of cereals	*Erysiphae graminis*	Cereals	Chlorotic or necrotic leaves, stems and heads covered with mycelium and spores of the fungus, often giving a white 'woolly' appearance.
Cankers, galls and malformations			
Clubroot of brassicas	*Plasmodiophora brassicae*	Brassicas	Wilting of leaves, swelling and distortion of roots, stunted growth; when severe roots rot and plant dies

* Many fungi cause more than one of the diseases (e.g. *Botrytis* spp. can cause blights and rots).

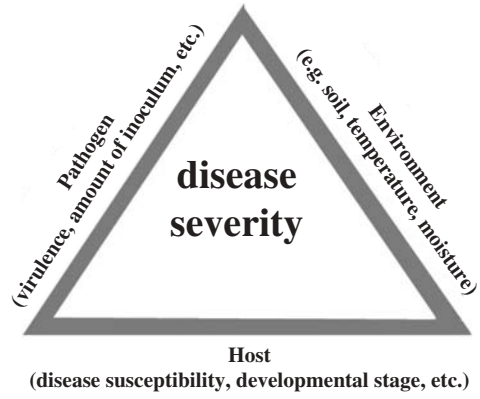

Figure 9.1 The disease triangle

9.3 Factors influencing disease development

The relationship between a phytopathogen and plant and the development of disease is influenced by the nature of the pathogen and host, and the prevailing environmental conditions; all three interact and form integral parts of the *disease triangle* (Figure 9.1). Each component represents one side of the triangle. For example, if environmental conditions are unfavourable for disease development, e.g. wrong temperature, wind or moisture conditions, then the environment side of the triangle would be shorter and therefore the overall disease level would be low or non-existent.

9.3.1 The pathogen

In order for disease to occur, a pathogen must be virulent towards, and compatible with, its host. The aggressiveness of a pathogen will influence disease development. Some pathogens have a broad host range, while other attack relatively few plant species. Some fungal species are comprised of *formae specialis* (f.sp.), each of which parasitize and cause disease of one or a small number of host plants. For example, many different f.sp. of *Fusarium oxysporum* cause wilt disease of many host species. Also, races may exist within a fungal species that differ in their pathogenicity towards different cultivars (or varieties or genotypes) of the same host species.

Infective propagules (e.g. fungal spores) must be present for disease to occur and to some extent the amount of inoculum influences disease development. The length of time that a pathogen is in contact with a host is also critical for disease development. The pathogen must also be able to compete with other organisms present on or in the plant.

9.3.2 The host

Disease development requires that the host plant be at a stage of development susceptible to infection. For example, damping-off disease only affects seedlings. Infection and colonization will not occur unless the host is susceptible to disease. Different plant species and even cultivars of the same species vary in their susceptibility to disease. For example, wheat cultivars or genotypes differ in their susceptibility to Fusarium head blight (FHB) disease caused by *Fusarium* spp. (Plate 7), which is a serious disease or cereals worldwide. Also, resistance to one pathogen does not mean immunity, i.e. plants may be susceptible to other fungal diseases (e.g. the FHB-resistant wheat cultivar Sumai 3 is susceptible to the potentially serious stem rust disease caused by *Puccinia graminis*). Plants may be disease tolerant; i.e. even though infected, they survive and grow and symptoms are not visible or are at a non-destructive level.

9.3.3 Environmental conditions

Temperature, wind, moisture, sunlight, nutrition and soil quality are environmental factors that have a major impact on disease development. Fungal pathogens differ in their optimal environmental conditions required for inoculum production, dispersal and disease development. Often, disease development by fungal pathogens requires a minimum exposure time to particular temperature and moisture combinations. For example, low relative humidity can reduce the development of powdery mildew disease of tomato caused by *Oidium neolycopersici*.

9.4 The disease cycle

The *disease cycle* describes the events that occur from initiation of disease to the dispersal of the pathogen to a new host plant. This is distinct from the *life cycle* of the pathogen or plant that describes the stage or successive stages in the growth and development of an organism that occur between the appearance and reappearance of the same state (e.g. spore or seed) of the organism. *Pathogenesis* describes events that occur in the disease cycle from infection to final host reaction.

Figure 9.2 depicts a generalized disease cycle. Inoculum is produced and disseminated, and after reaching its target host plant tissue (inoculation) it penetrates the host. The type and mode of production of inoculum (e.g. sexual and asexual spores, resting spores, mycelium) and the method of dissemination (e.g. wind, water, insect) depends on the particular pathogen. For many important plant pathogens, a sexual stage has not been identified. Penetration is through wounds or natural plant pores (e.g. stomata), and some fungi produce special-

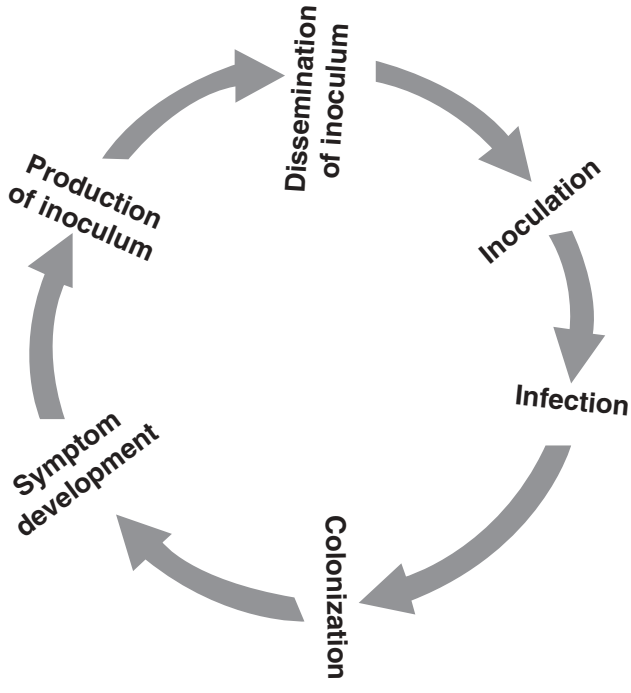

Figure 9.2 A generalized fungal disease cycle

ized penetration structures called appressoria (singular = appressorium) (Figure 9.3). Having penetrated its host, the fungus then grows within plant tissue (infection). The incubation period defines the period between inoculation and infection. As it grows within the host, it utilizes the plant's cellular resources as a nutrient source and the damage inflicted on the plant is manifested as disease symptoms. The latent period defines the period between infection and symptom development. The pathogen forms survival structures such as spores that are then disseminated. During the plant pathogenesis, some diseases only involve one such disease cycle (primary cycle), while others have the potential to do more damage as they involve secondary or multiple cycles of disease.

9.5 Genetics of the plant–fungal pathogen interaction

Fungal genes encode proteins that make the fungal specific and virulent to particular hosts, and hosts in turn have genes that make it susceptible or resistant to a particular fungus. The gene-for-gene hypothesis explains the interaction between cognate sets of pathogen and host genes. The host has dominant genes for resistance (*R*) and recessive genes for susceptibility (*r*). The pathogen has recessive genes for virulence (*a*) and dominant genes for avirulence or inability

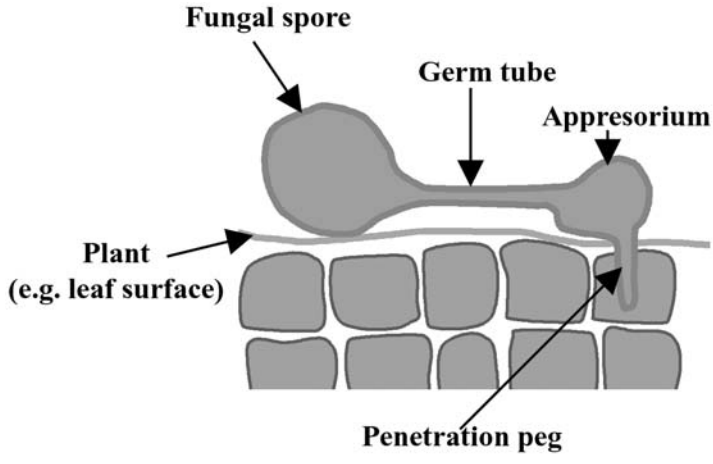

Figure 9.3 Illustration of fungal penetration of a host plant by means of a specialized appresorium and penetration peg

Table 9.2 The gene-for-gene interaction between fungal pathogens and plants*

Host → Pathogen ↓	Resistant (*R*)	Susceptible (*r*)
Avirulent (*A*)	*AR* (incompatible)	*Ar* (compatible)
Virulent (*a*)	*aR* (compatible)	*ar* (compatible)

* Incompatible interactions result in no disease, i.e. plant is resistant to that pathogen, while compatible interactions result in disease, i.e. plant is susceptible (Flor, 1956). Only *AR* interactions are resistant – in all other cases, disease will occur.

to infect (*A*). The interaction between specific sets of pathogen avirulence/virulence and host resistance/susceptibility genes determines whether disease develops. Table 9.2 explains the possible interactions between such plants and pathogens. Of the possible combinations of genes, only *AR* interactions are resistant, and in all other cases disease will occur. *R* genes encode receptors that interact with *A* genes. Many avirulence genes (*A*) act as virulence genes (*a*) in the absence of the corresponding host resistance gene (*R*).

9.6 Mechanisms of fungal plant parasitism

Fungi parasitize plants using physical, chemical and biological means. In doing so they adversely affect photosynthesis, translocation of water and nutrients, respiration, transcription and translation in host tissue.

9.6.1 Mechanical means of parasitism

Mechanical means of plant parasitism include the adherence to host tissue and forceful penetration; chemical and biological molecules often facilitate such steps. Biotrophic plant pathogens have evolved specialized *appressoria*, which are highly organized enlarged ends of hyphae (Figure 9.3). Once the hypha senses an appropriate site, it enlarges and adheres to the leaf surface. This adherence is necessary to support the amount of mechanical force used to penetrate into the plant via a hypha called a penetration peg.

9.6.2 Pathogen metabolite-mediated parasitism

Enzymes

Fungi produce a range of enzymes that facilitate host plant infection and colonization by degrading the cellular and intercellular constituents of plants (certain fungal pathogens also produce non-enzymatic proteins that inhibit the activity of plant enzymes involved in the host defence response). The cuticle forms a continuous layer over aerial plant parts and is an important barrier against pathogens and other stresses. Cutin is the major structural component of the cuticle barrier and some fungi secrete cutinases that hydrolyse ester linkages between cutin molecules. In doing so, they, release monomers as well as oligomers, thus breaking the integrity of the cuticle barrier and facilitating plant parasitism.

The plant cell wall presents a complex and important physical barrier against invading fungi. Pectinaceous substrates form part of the cell wall and are usually a major constituent of the middle lamella that adheres to adjacent plant cells. They are polysaccharides, consisting mostly of galacturonan molecules interspersed with rhamnose molecules and side-chains of galacturonan and other sugars. There are many pectin-degrading enzymes that attack different parts of the polysaccharide, including pectin esterases, polygalgacturonases and pectate lyases, resulting in a general disintegration of the host tissue.

Cellulose, the most abundant natural polymer and a component of plant cell walls, consists of repeating units of glucose molecules, and these chains cross-link to form fibrils embedded in a matrix of other polymers such as pectin and lignin. Fungi often produce cellulose-degrading enzymes, or cellulases, in the latter stages of plant parasitism. Different cellulases attack the cross-linkages, and the polymeric, or degraded oligomeric or dimeric cellulose chains, in a series of steps, resulting in the degradation of cellulose into small oligomers of the monomer glucose. Hemicellulose represents a complex mix of polysaccharides that forms part of the plant cell wall matrix and is also present in the middle lamella. The primary polysaccharide component is xyloglucan, and others

include glucomannan, galactomannans and arabinogalactans. Fungi produce an array of hemicellulose-degrading enzymes necessary for complete hydrolysis of the substrate. Thus, cellulases and hemicellulases reduce the host cell wall integrity.

Lignin is the second most abundant natural polymer on earth, being ubiquitous in monocots and dicots and a major component of woody plants. The basic lignin polymer consists of chains of substituted phenylpropanoid molecules, and this polymer is substituted with a variety of side chains. Certain fungi, mostly basidiomycetes, can degrade lignin. The white-rot fungi produce ligninase enzymes that degrade most of the lignin in the world.

Various other fungal enzymes facilitate plant pathogenesis. These include protein-degrading proteases and lipid and starch-degrading enzymes. Proteases can interfere with membrane integrity by degrading the protein component of the plasma membrane. Some enzymes degrade plant compounds involved in the host defence response.

Polysaccharides, toxins and growth regulators

Certain fungi also produce polysaccharides that facilitate host colonization or deactivation of the host defence response. Some fungi produce toxins that seriously damage or kill the host cells, and they range from low-molecular-weight metabolites to proteins. Examples of fungal toxins, their producers and the associated diseases and hosts are listed in Table 9.3. Fungal toxins may be host- or non-host-specific. Host-specific toxins are usually required for pathogenicity, but are only toxic to the host plants of a pathogen, and show no or low toxicity to other plants. In contrast, non-host-specific toxins affect a wide range of host plants, and may act as virulence factors increasing the extent of the disease, but are not essential for disease development. In addition to being phytotoxic, some non-host-specific toxins are also classified as mycotoxins, i.e. harmful to human and animal health (e.g. trichothecenes, fumonisins and fusaric acid). Fungal toxins may cause visible symptoms such as wilting, chlorosis, stunting (Plate 8), etc., or they may operate at the biochemical level and not cause visible effects.

Increased levels of growth regulators or hormones are often associated with diseases of plants; whether these originate from the fungus or the plant is often unclear. Growth regulators affect plant growth in many ways. Increased cytokinin production is associated with tumour formation (e.g. clubroot of brassicas caused by *Plasmodiophora brassicae*, Plate 9) and peach leaf curl caused by *Taphrina deformans*. The pathogen *Gibberella fujikuroi* secretes gibberellins, causing 'foolish seedling' disease of rice characterized by overgrown, weak, spindly plants. Ethylene is a volatile hormone and increased levels are associated with fungal diseases, but its origin is often unclear, i.e. fungus or plant.

Table 9.3 Examples of fungal toxins, their specificity, producer fungi and associated diseases

Toxin	Producer fungi	Associated diseases
Host-specific		
Victorin	*Cochliobolus victoriae*	Victoria blight of oats
HS toxin	*Bipolaris sacchari*	Eyespot disease of sugar cane
HC toxin	*Cochliobolus carbonum* race 1	Northern leaf spot and ear rot of maize
T toxin	*Cochliobolus heterostrophus* race T	Southern corn (maize) leaf blight
AK toxin, AF toxin, ACT toxin, AM toxin, AAL toxin, ACR(L) toxin	*Formae speciales* of *Alternaria alternata*	Diseases of Japanese pear, strawberry, tangerine, apple, tomato, rough lemon, respectively
PM toxin	*Mycosphaerella zeae-maydis*	Yellow leaf blight of corn (maize)
Peritoxin (PC toxin)	*Periconia circinata*	Sorghum root rot
Ptr ToxA, Ptr ToxB	*Pyrenophora tritici-repentis*	Tan spot of wheat
Non-host-specific		
Tentoxin	*Alternaria* spp.	Various
Solanopyrones	*Alternaria solani*	Early blight of potato
	Didmella rabiei (anamorph: *Ascochyta rabiei*)	Blight of chickpea
Trichothecenes	*Fusarium* spp.	Head blight, seedling blight and root rot of wheat
Fumonisins	*Gibberella fujikuroi* (anamorph: *Fusarium moniliforme*)	Ear rot of maize
	Anternaria alternata	
Enniatins	*Fusarium* spp., e.g. *F. avenaceum*	Dry rot of potato
Fusicoccin	*Fusicoccum amygdali*	Fusicoccum canker of peach, nectarine and almond
Sirodesmin PL, depsipeptide HST, phomalide	*Leptosphaeria maculans*	Blackleg of crucifers
Oxalic acid	*Sclerotium, Sclerotinia*	
Fomannoxin	*Heterobasidion annosum*	Root and butt rot of conifers
Cerato-ulmin	*Ophiostoma novo-ulmi*	Dutch elm disease
Cercosporin	*Cercospora* spp.	Various

9.7 Mechanisms of host defence

Different host species and genotypes of a host plant species vary in their susceptibility to fungal diseases and non-hosts are disease resistant. This variation in disease resistance is due to structural and/or metabolic differences amongst plants. Table 9.4 highlights some of the preformed and induced structural and metabolic defence strategies used by different plants or plant cultivars to combat fungal diseases. The host defence response may be localized or systemic.

Plant disease resistance (*R*) genes encode receptors that interact with fungal avirulence (*A*) genes. The protein products of *R* genes have to both perceive and activate signals, i.e. to recognize the pathogen signal (*A* gene) and activate a plant defence response. The majority of *R* genes encode structurally related proteins with motifs that target their intracellular or extracellular localization (leucine-rich repeat or LRR motifs and nucleotide binding (NB) motifs). Having perceived the pathogen, products of the *R* genes in disease-resistant hosts induce an active host defence response.

Plant defences vary in their time of activation in response to fungal attack. Rapid cellular defence responses include an oxidative burst, callose synthesis and deposition, generation of nitric oxide (NO) and cross-linking of cell wall proteins. Nitric oxide and oxidative-burst-generated O_2^- and H_2O_2 possess antimicrobial activity. Callose, a 1,3-linked glucan that is deposited as papillae (localized wall appositions), together with the cross-linked cell wall proteins, increases the cellular resistance to fungal penetration.

Slower responses include the production of phytoalexins, pathogenesis-related proteins (PRPs) and hydroxyproline-rich glycoproteins (HGRPs), and the induction of lignification, suberization, the hypersensitive response (HR) and induced or systemic acquired resistance (ISR or SAR). Phytoalexins are low-molecular-weight compounds that, non-specifically, inhibit a wide range of organisms. HGRPs are a constitutive part of cells, but can also be induced by wounding and infection. Although it is known that they play a role in cross-linking of cell wall proteins, much remains to be discovered as to their role in plant defence. Lignin and suberin are both constitutive components of healthy plant cell walls; enhanced synthesis and deposition of these molecules in response to infection is thought to increase the resistance of cell walls to penetration and degradation. HR involves a genetically programmed suicide of the cells surrounding the infecting fungus and both O_{2-} and H_2O_2 may participate in this response. The HR is particularly debilitating to biotrophic and hemibiotrophic pathogens that require living plant tissue to survive; its role in response to necrotrophic pathogen invasion is not so clear. Also, HR is not an obligatory component of disease resistance and is absent, or occurs as a late event, in some resistant responses of plants to pathogens.

SAR describes the resistance that develops in plants at a distance from the initial infection point, i.e. a process that is induced by infection and immunizes

Table 9.4 Preformed and induced defence strategies used by plants to combat fungal diseases

Type	Time of formation	Examples
Structural	Preformed	Thick wax or cuticle layer covering the epidermis
		Thick and/or tough cell walls (e.g. highly lignified walls impede pathogen advancement)
		Size, shape and location of stomata
	Induced	Cork cells impeding pathogen advancement
		Isolation of infected area by formation of abscission layers
		Tylose formation in xylem vessels (blocking pathogen advancement through the vascular system)
		Gums deposited in intra- and inter-cellular spaces forming an impenetrable barrier to the pathogen
		Morphological cell wall alterations (e.g. lignification)
		Hypersensitive response (destroying plant cells in contact with the pathogen and thus starving the fungus of nutrients)
		Fungitoxic exudates
Metabolic	Preformed	Lack of elicitor production
		Lack of host receptor production
		Low content of essential pathogen nutrients
		Antifungal phenolics, tannins, saponins or antifungal enzymes (e.g. glucanases and chitinases)
		Plant defence proteins
	Induced	Formation of callus and cork cells around infection
		Production of phenolics and phytoalexins (that may be hypersensitive reaction-associated metabolites)
		Transformation of non-toxic to toxic compounds e.g. phenolics, cyanides
		Production of fungal-degrading enzymes
		Production of compounds that inhibit pathogen enzymes
		Detoxification of pathogen toxins
		Complexing of plant substrates to resist fungal enzyme attack
		Metabolites involved in localized and systemic induced resistance

the plant against future pathogen attack. A salicyclic acid-dependent process mediates the SAR response and mitogen-activated protein kinase (MAPK) signal transduction cascades have been implicated as negative regulators of salicyclic acid accumulation and inducers of resistance. PRPs are acidic, protease-resistant proteins, some of which possess antifungal activity (e.g. glucanases and chitinases that degrade the chitin component of fungal cell walls). Some increase the permeability of fungal cell membranes (thionins), or affect membrane transport (defensins) or inactivate fungal ribosomes (ribosome-inactivating proteins). SAR results in the formation of plant PRPs. In response to infection, PRPs are known to accumulate in resistant plants in tissue distant from the infection point and their accumulation is correlated with the development of SAR. ISR differs from SAR, at least in some systems, in that it is mediated by a jasmonic acid/ethylene-dependent process and is not accompanied by the accumulation of PR proteins.

9.8 Disease control

9.8.1 Cultural practices that aid disease control

Removal of crop debris, stubble or weeds helps control fungal diseases, as many causal organisms survive and overwinter on these materials. The most famous example of a fungus with an alternative host was that of *Puccinia graminis* f. sp. *tritici,* the cause of the devastating black stem rust disease of wheat. This fungus completes its life cycle on a barberry bush. For this reason, barberry bushes are eradicated from the USA. Plant rotation in cropping systems will help prevent inoculum build-up provided that all hosts are not susceptible or do not act as alternative hosts for prevalent diseases. Good sanitary practices will help prevent dissemination of inoculum by mechanical means such as contami-nated equipment. The date and method of sowing and the quality of the seedbed can influence disease development. As a general rule earlier-sown seeds tend to suffer more disease problems than those sown late.

9.8.2 Fungicidal control of plant pathogens

There is a huge array of fungicides on the market for controlling fungal diseases. Depending on the disease and the host, fungicides are applied as a fumigant, spray, dust, paint, soil treatment, etc. Fungicides are classified in several different ways according to how they penetrate plant tissue, what effect they have and according to their mode of action. Fungicides may act in the following ways.

- *Protectant*: do not penetrate the plant, but affect pathogen viability and germination on the surface of the host plant. Such fungicides must adhere to the plant surface and resist weathering and are most effective when applied as a preventative measure, i.e. before plant inoculation with the fungus.
- *Systemic*: can act on the plant surface and be translocated throughout the plant vascular system to kill the fungus. Systemic fungicides may also exhibit translaminar movement within the leaves.
- *Eradactive*: They are applied post-infection and act on contact by killing the organism or by preventing its further growth and reproduction.

Strobilurins represent a relatively new class of broad-spectrum fungicides that exhibit translaminar properties. Some are systemic and some are mesostemic, i.e. act on plant surface, penetrate plant tissue, show translaminar movement, are absorbed by the waxy layer and exhibit vapour movement and redistribution within the crop canopy.

Fungicides are further classified into numerous groups according to their target sites. For example, systemic sterol biosynthesis inhibiting fungicides (SBIs) interfere with ergosterol production in fungi, and hence with the integrity of the cell membrane. Strobilurins disrupt the production of ATP. Some chemicals induce systemic disease resistance in the plant (defence activators).

Many fungal pathogens have evolved resistance to fungicides by changing the target site of the fungicide. For example, many target pathogens have evolved resistance to the broad-spectrum methyl benzimidazole carbamate (MBC) fungicides. These fungicides inhibit tubulin assembly (and hence mitosis) and a point mutation in the β-tubulin gene of some fungi renders them unaffected by such fungicides.

9.8.3 Host resistance to disease

If disease-resistant cultivars of a host have been identified, the resistance can be introduced into host cultivars that possess other desirable characteristics such as high yield. If the resistance is already in a cultivar of the required plant species, or in closely related species, this may be achieved by conventional plant breeding techniques. Also, identified gene(s) associated with resistance can be incorporated into the required plant via genetic engineering. Candidate genes for enhancing disease resistance include those that code for proteins that are either antifungal or regulate pathways involved in the host defence response (see section 9.7). New molecular technologies such as enabling the plant to turn off or silence genes involved in host susceptibility or pathogen virulence (RNA silencing) will increase the number of potential target genes for genetic engineering.

9.8.4 Biological control of fungal pathogens

Biological control uses organisms, i.e. biocontrol agents (BCAs), to directly or indirectly control fungal diseases of plants. Most BCAs used to control fungal diseases of plants are environmentally acceptable and non-plant-pathogenic bacteria or fungi. Biological control is not a new phenomenon as it occurs naturally. For example, the natural microbial population of certain soils suppresses the development of wilt diseases caused by vascular wilt fungi such as *Fusarium oxysporum*. Also, organic matter soil additions (e.g. compost) provide nutrients for, and therefore increase, the resident microbial population, some of which may be disease suppressive. Biological control can therefore be attempted either by the introduction of foreign organisms or, less commonly, manipulation of the natural microbial population to enhance the activity of resident disease-suppressive organisms.

There are three means by which microbes can inhibit the development of a fungal disease (Figure 9.4).

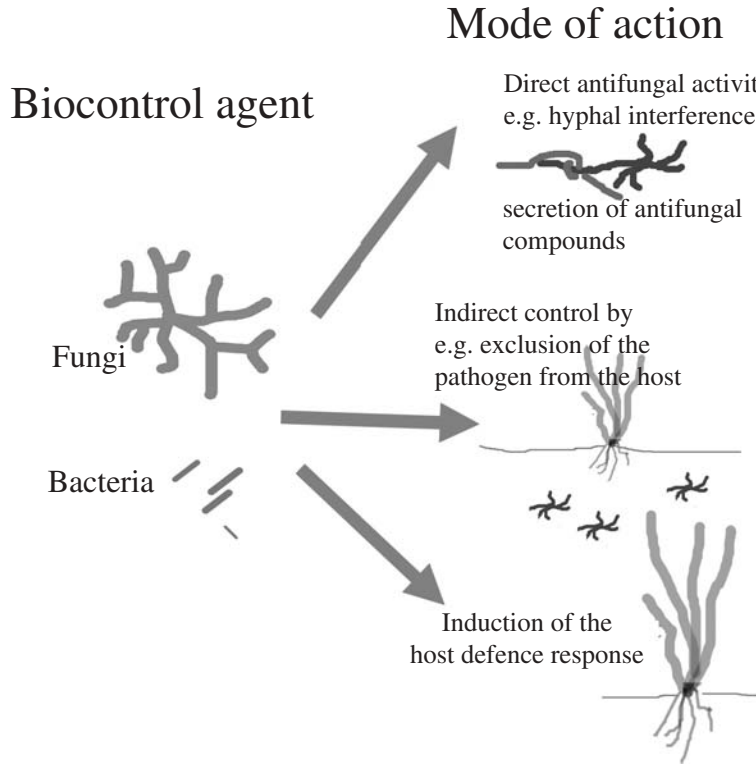
Mode of action
Biocontrol agent
Direct antifungal activity, e.g. hyphal interference
secretion of antifungal compounds
Indirect control by e.g. exclusion of the pathogen from the host
Fungi
Bacteria
Induction of the host defence response

Figure 9.4 Means by which biocontrol agents can inhibit the development of a fungal disease

- *Direct*: the BCA suppresses the pathogen by one or more of the following means: hyphal interference, secretion of antifungal compounds (e.g. antibiotics) or enzymes. The fungus *Trichoderma harzianum* is used for the biocontrol of various fungi, which it achieves by means of hyphal interference and the secretion of hydrolytic enzymes that attack the pathogenic fungi.
- *Indirect*: the BCA physically excludes the pathogen from contact with its host or out-competes the pathogen for nutrients in a particular niche. In the rhizosphere, hypovirulent (non-virulent) strains of *F. oxysporum* can outcompete some virulent strains associated with certain vascular wilt diseases.
- *Induction of host defence*: the BCA induces the host defence mechanisms. For example, *Pseudomonas* spp. induce SAR to vascular wilt fungi in various hosts.

An effective BCA must be produced, formulated and delivered in such a way that it can reach its target ecological niche in a viable and active form. Although many potential BCAs are effective under experimental conditions, many prove ineffective under field conditions. Many potential BCAs are isolated from a habitat similar to that in which they will be applied (e.g. Plate 10). Table 9.5 lists some of the commercially available BCAs for the control of fungal diseases.

9.9 Disease detection and diagnosis

Host disease symptoms can help a preliminary disease diagnosis and give the first clue as to the causal organism. Isolation and purification of the pathogen on culture media and subsequent macroscopic and microscopic analysis of fungal structures (e.g. hyphae, spores, resting structures) may confirm its identity.

These traditional diagnostic techniques, although invaluable, often require a considerable amount of time and plant pathology expertise. Also, the disease will have advanced to the stage where symptoms are expressed before detection and diagnosis can occur, and at this stage control measures adopted may be reduced in their efficacy. These disadvantages have led to the development of more rapid diagnostic techniques for many diseases, that can often detect as little as a few propagules of the pathogen in asymptomatic plant material. Such techniques include enzyme-linked immunosorbent assays (ELISA) using antibodies specific to the particular pathogen, and the more commonly used pathogen-specific polymerase chain reaction (PCR) tests that detect DNA sequences specific to the pathogen.

Table 9.5 Examples of commercially available biocontrol agents for the control of fungal diseases

Biocontrol agent	Commercial product trademarks	Disease
Bacteria		
Bacillus spp.	Companion	Soil-borne diseases of greenhouse and nursery crops
Bacillus subtilis QWT713	Serenade	Various diseases including powdery and downy mildews of vegetables, grapes, hops and other crops
Burkholderia cepacia	Intercept, Deny	Damping-off and rot diseases caused by soil-borne *Rhizoctonia*, *Pythium* and *Fusarium* spp.
Candida oleophila I-182	Aspire	Diseases of citrus and pome fruit caused by *Botrytis* and *Penicillium* spp.
Pseudomonas aureofaciens	BioJect Spot-Less	Anthracnose, damping-off and pink now mould diseases of turfgrass and other hosts
Pseudomonas chlororaphis	Cedomon	Seed-borne and foliar diseases of cereals caused by various pathogens
Fungi		
Ampelomyces quisqualis isolate M-10	AQ10 Biofungicide	Powdery mildew of apples, cucurbits, grapes, ornamentals, strawberries, and tomatoes
Fusarium oxysporum (non-pathogenic)	Biofox C, Fusaclean	Fusarium wilt of basil, carnation, cyclamen, tomato
Gliocladium virens GL-21	Soilgard	Damping-off and root rot diseases of ornamentals and greenhouse and nursery food crop plants caused by e.g. *Rhizoctonia solani* and *Pythium* spp.
Trichoderma spp.	Various, e.g. Binab, Supresivit PlantShield, T-22 Planter box	Soil-borne fungi causing rots, damping off and wilts of various hosts including fruit, ornamentals, turf and vegetables

9.10 Vascular wilt diseases

Wilt diseases arise from a water deficiency in plant foliage. This wilting results because fungus infection and colonization of the host results in a blocking of the xylem vessels and consequent inhibition of water translocation. The fungus may directly or indirectly mediate the blocking of the xylem vessels.

- *Direct*: the fungus colonizes the xylem vessels and physically blocks water movement within the vessel.
- *Indirect*: the host responds to fungal infection by secreting substances that block its xylem vessels in order to limit the spread of the pathogen

Fusarium and *Verticillium* species cause vascular wilt diseases of a range of host plants; other fungal wilts of economic significance include Dutch elm disease and oak wilt (Table 9.1).

9.10.1 Fusarium wilts

Vascular wilt caused by soil-borne *Fusarium* species, especially *Fusarium oxysporum*, is common in many parts of the world. It produces asexual macroconidia (characteristic of *Fusarium* species) (Plate 11) and resting spores called chlamydospores (hyphal swellings) and smaller asexual microconidia characteristic of select *Fusarium* species. Mycelia are yellow/red pigmented when grown in culture on potato dextrose agar (Plate 11(b)). The sexual state of this fungus, if it exists, has not yet been found.

Fusarium oxysporum attacks, and causes serious economic losses of most vegetable and flower crops, cotton, tobacco, banana, plantain, coffee, turfgrass, ginger and soybean, in warm and temperate climates. More than 120 *formae speciales* (f. sp.) have been identified (e.g. wilt disease of tomato is caused by *F. oxysporum* f. sp. *lycopersici*, while wilt disease of banana is caused by *F. oxysporum* f. sp *cubense*). Some f. sp. are primarily associated with root rots, foot rots or bulb rots of plants, rather than with vascular wilt disease. Within many f. sp., *F. oxysporum* isolates are further classified into races according to their virulence against different host genotypes.

Plate 11(c) depicts the typical disease symptoms associated with vascular wilt disease of tomato plants caused by *F. oxysporum* f. sp. *lycopersici*. In addition to wilting, other symptoms associated with this disease include vein clearing in younger leaflets in the early stages of disease, epinasty, stunting and yellowing of older leaves.

Figure 9.5 depicts the typical wilt disease cycle for *Fusarium oxysporum*. Conidia of the fungus germinate in response to root exudates, producing penetration hyphae that attach to the root surface and penetrate it directly. The

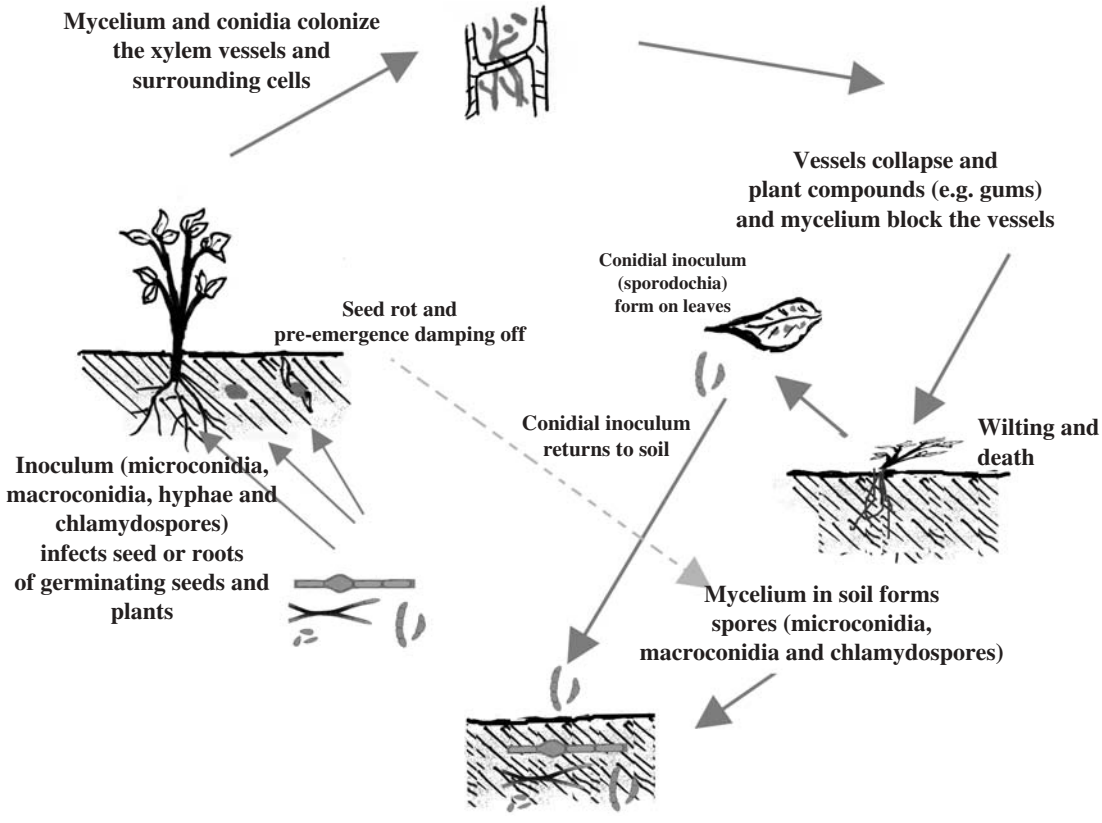
Mycelium and conidia colonize the xylem vessels and surrounding cells
Vessels collapse and plant compounds (e.g. gums) and mycelium block the vessels
Conidial inoculum (sporodochia) form on leaves
Seed rot and pre-emergence damping off
Conidial inoculum returns to soil
Wilting and death
Inoculum (microconidia, macroconidia, hyphae and chlamydospores) infects seed or roots of germinating seeds and plants
Mycelium in soil forms spores (microconidia, macroconidia and chlamydospores)

Figure 9.5 Simplified Fusarium wilt disease cycle (adapted from Agrios, 1988)

mycelium then advances inter-cellularly through the root cortex and enters the xylem vessels through the pits. Within the xylem vessels, the fungus produces microconidia that are carried upwards in the sap stream. Upon germination, microconidia penetrate the upper wall of the vessels, producing more microconidia in the next vessel. The plant is subjected to water stress and subsequently wilts due to both the accumulation of fungal mycelium and/or toxin production, and the host defence responses induced as a result of pathogen attack. Once the plant is killed, the fungus spreads to invade the parenchymatous tissue and sporulates profusely on the plant surface. These conidia can be returned to the soil where they can survive for extended periods. Conidia are disseminated to new plants or areas by wind, water etc.

Plant infection and colonization by *F. oxysporum* is facilitated by the pathogen's perception of signals present in root exudates, hyphal adherence to the root surface, secretion of a battery of cell wall degrading enzymes and the pathogen's possession of multiple mechanisms of overcoming host defence

mechanisms. Root exudates elicit a fungal response and evidence suggests that cellular signal transduction cascades mediate this response. Host molecules that activate such cascades and the mechanisms by which the fungus perceives such signals, i.e. the signal receptors, are not yet known. *Fusarium oxysporum* secretes an array of cell-wall-degrading enzymes that may contribute to infection and colonization (e.g. polygalacturonases, pectate lyases, xylanases and proteases). Pectate lyases are thought to play an important role in the virulence of Fusarium wilt fungi. Plants have evolved a number of physical, chemical and biological defence strategies against *Fusarium* wilt disease, including the deposition of callose adjacent to infected cells, the 'blocking off' of colonized vessels by gels, gums and tyloses, the synthesis of fungitoxic compounds and antifungal enzymes.

Control of Fusarium wilt is difficult due to the soil-borne nature of the pathogen and its ability to persist for extended periods in the absence of a host. Disease control is usually achieved via an integrated pest management system, i.e. the combined use of one or more of the following – cultural practices, resistant host genotypes, fungicides and biological control agents. Cultural practices that help control this disease include rotating land with non-hosts such as cereals (avoiding solanaceous crops such as potato and tomato), removal of crop debris, non-excessive irrigation and the maintenance of vigorous plants via fertilizer application.

Traditionally, chemical control of Fusarium wilt was often attempted by soil fumigation with methyl bromide to kill the soil-borne conidial inoculum. In more recent years, environmentally friendly alternatives have been revisited such as soil steam sterilization of limited amounts of substrates (e.g. for glasshouse use) and reduction of seed-borne inoculum using hot-water treatment. Fungicide seed treatment can protect the plant during the early stages of establishment. Certain systemic fungicides can protect the plant at later developmental stages but their use is not encouraged for food plants. Biological control of Fusarium wilt has received a lot of attention in recent years as an attractive means of disease control. Currently, potential biocontrol organisms for controlling Fusarium wilt are on the market (Table 9.5) and others are being developed (e.g. non-pathogenic strains of *F. oxysporum*).

9.10.2 Other wilts of economic significance

Verticillium wilt

Verticillium wilt is commonly caused by the soil-borne *Verticillium albo-atrum* and *V. dahliae*. These lack known sexual reproductive structures (deuteromycetes) and the asexual conidia are produced. *V. albo-atrum* grows best at 20–25 °C, while *V. dahliae* grows better at 25–28 °C. Verticillium wilt affects a wide array of vegetables, flowers, field crops, fruit trees, roses and forest

trees. Both *V. albo-atrum* and *V. dahliae* exist as various strains or races whose host range, virulence and other characteristics vary considerably. The symptoms and disease cycle of Verticillium wilt are similar to those of Fusarium wilt. Symptoms, often not obvious until either dry weather or later in the growing season, include wilting, stunting, vascular discolouration, leaf epinasty and chlorosis and necrosis of leaves. Compared to Fusarium wilt, Verticillium wilt symptoms develop more slowly, at lower temperatures, often only develop on one side of a plant and are usually confined to lower plant parts. Both *Verticillium* species can be spread in contaminated seed, propagative plant parts and by wind, water and soil, and can overwinter and persist as mycelium in perennial hosts, plant debris and vegetative propagative parts. As with Fusarium wilt, control is usually based on an integrated pest management strategy similar to that described above for Fusarium wilt. Resistant or partially resistant cultivars of some susceptible plant species are available and durable resistance has been incorporated into many commercial tomato cultivars.

Dutch elm disease

Dutch elm disease has devastated elm populations worldwide over the last 100 years; the devastation continues, particularly in the USA. Two species of the ascomycetous heterothallic *Ophiostoma* genus are responsible for the disease, *O. ulmi* and the more aggressive *O. novo-ulmi* (the causal organism was traditionally called *Ceratocystis ulmi*). Disease symptoms include wilting, yellowing/browning of leaves of individual branches or of the entire tree (Plate 12) and the formation of brown or green streaks in the infected sapwood underlying the bark. The disease is most destructive when trees are infected early in the growing season. The fungus overwinters as a saprophyte in dying or dead elm trees or logs as either mycelium or conidia-bearing coremia. The fungus is spread by emerging adult elm bark beetles that become coated with sticky conidia. These beetles wound and colonize other elms, to which they transfer their conidial loads (the fungus can also spread through grafted root systems). The conidia germinate and rapidly parasitize and colonize the wood until they reach the large xylem vessels of the spring wood, where they may produce more conidia that are carried up in the sap stream and initiate new infections. Control of Dutch elm disease spread may be facilitated by proper sanitation, removal of localized branch infections by pruning, avoidance of contact between healthy and infected roots and immediate removal of infected elms. Root injection with systemic fungicides may give protective or short-term curative control, but this may be expensive. Elimination of the disease vector, the elm bark beetles, can be attempted (using pesticides a pathogenic nematodes or trapping using pheromones), but, in the past, this has not proved to be very effective in controlling Dutch elm disease. Control via innate host resistance is unlikely, as most elm species are susceptible to this disease.

Oak wilt disease

The ascomycetous fungus *Ceratocystis fagacearum* causes oak wilt disease. Infected trees rapidly wilt from the top of the canopy downward and trees usually die within two months. Leaf symptoms of oak wilt include wilting, clearly delineated browning and defoliation. These symptoms are sometimes accompanied by a brown discoloration of the sapwood underlying the bark of symptomatic branches. Red oaks are very susceptible to the disease; members of the white oak family are generally more resistant. The fungus overwinters as mycelium in living infested trees and as fungus pads on dead trees. Insects such as sap and bark-feeding beetles can spread the fungus. Control of oak wilt disease spread may be facilitated by means similar to those described above for Dutch elm disease.

9.11 Blights

Fungal blight diseases of plants are characterized by a rapid browning and death of the plant leaves, branches, twigs and floral organs. Table 9.1 lists some of the economically important blights caused by plant-pathogenic fungi (many of these fungi cause additional disease symptoms, such as rots etc., on these and other hosts). The most famous example is late blight of potatoes.

9.11.1 Late blight of potatoes

Phytophthora comprises a genus of fungi that cause a range of disease symptoms on different host plants. Of the different species, *Phytophthora infestans* is best known as it is the causal agent of late blight of potatoes and tomatoes. This is an ubiquitous disease in most potato-growing regions of the world and is very destructive in the potato-growing regions of Europe and the USA. This disease precipitated the Irish potato famine in the 19th century.

Phytophthora infestans is a heterothallic oomycete that produces motile zoospores (that have two flagella) in lemon-shaped sporangia that form at the top of sporangiophore branches (Plate 13(a)). At higher temperatures (>15 °C) sporangia can germinate directly by producing a germ tube, while at lower temperatures (<12–15 °C) sporangia germinate almost entirely by means of zoospores. Sexual reproduction requires the mating of an A1 and A2 type of the fungus whose hyphae on contact differentiate to form an antheridium and oogonium. The antheridium fertilizes the oogonium (a process called karyogamy), which develops into a thick-walled oospore that germinates by means of a germ tube (Plate 13(b)). This germ tube then forms a sporangium or, occasionally, directly forms a mycelium.

This disease affects stem, leaf and tuber tissue. Leaf symptoms of late blight of potato include water-soaked spots on lower leaves that, under moist conditions, subsequently enlarge to form brown areas (Plate 13(c)). On the underside of these infected leaves a white woolly fungal growth appears and soon all the leaflets on the leaf die. Under prolonged periods of wet or damp weather, all aerial plant parts blight and rot, giving a foul odour. Affected tubers have brown/black blotches on their surface, and internally they exhibit water-soaked dark-brown rotted tissue (Plate 13(d)). Such tissue may be colonized by secondary soft-rotting invaders (bacteria and fungi), resulting in a foul odour.

The pathogen overwinters as mycelium in infected potato tubers. Overwintering mycelium infects and spreads though the cortical region of the stems of plants arising from infected tuber seed, and later colonizes the pith cells of the stem. Once the mycelium reaches the aerial plant parts it produces sporangiophores that protrude through the leaf and stem stomata and project into the air. Sporangia are produced on the sporangiophores and, when mature, they are released into the air or are dispersed by rain. These sporangia land on potato leaves or stems where they form appresoria, and under moist conditions they germinate and the arising penetration peg penetrates the plant and causes new infections. The penetrating hypha grows intercellularly, and the resulting mycelium penetrates and forms intracellular haustoria, i.e. hyphae that enter and draw nutrients from the host cells. These colonized cells die and the disease spreads to fresh tissue. A large number of these asexual generations and new infections can occur in a short time period. These infections result in the blighting and premature death of foliage and a reduction in tuber yield. Tuber infection begins when, under wet conditions, sporangia are washed downwards from infected foliage into the soil, releasing zoospores that reach the tubers near the surface of the soil. Zoospores germinate and the germ tube penetrates the lenticels or wounds on tubers. The fungus colonizes the tuber cells by growing intercellularly and producing haustoria within the cells. Infected and colonized tuber tissue decays and rots, either in the field or in storage. Also, seemingly healthy tubers inoculated with sporangia from soil can become infected and rot during storage.

Relatively warm days and early evenings promote infection and sporulation; lowering of temperature during the night induces dew formation and zoospore production; gradually increasing temperature during the day promotes zoospore encystment and penetration. Infection and colonization of potato by *P. infestans* is facilitated by adhesion of the pathogen to the host surface, penetration and infection via physical force, degradative enzymes and other virulence molecules, and suppression of host defence responses. Zoospores of other *Phytophthora* species exhibit electrotactic swimming towards weak electric fields generated by plant roots, chemotaxis (attraction towards plant-derived compounds), and chemotrophic and contact-induced responses.

Control of late blight of potato has received much attention. It is generally attempted by an integrated approach involving cultural, chemical and disease-

forecasting control strategies. Proper sanitation, including the use of disease-free seed and removal of plant debris and volunteer potato plants, helps control late-blight disease. Regular fungicide applications are often used as a preventative measure under late-blight-favourable conditions, i.e. wet or humid weather and cool nights. Disease forecasting systems are widely used to indicate environmental conditions conducive to blight. Advanced systems take into account the resistance of the potato cultivar to late blight, the effectiveness of the fungicide and in some cases also the local disease risk. Most commercial cultivars are susceptible to late-blight disease of potato. However, some offer partial resistance to the disease and research is continuing to develop new cultivars with enhanced disease resistance via both traditional breeding and transgenic approaches. Genes conferring resistance to late blight are known to be present in both wild and cultivated potato germplasm. The potential of controlling late blight using biological control methods is being extensively investigated. Isolates of several fungal species (e.g. *Aspergillus, Fusarium, Mucor, Penicillium, Rhizoctonia* and *Trichoderma*) and bacterial species (e.g. *Bacillus subtilus*) have shown promise for disease control and some have been commercialized.

9.12 Rots and damping-off diseases

Many fungi cause rots of a wide variety of plants. These fungi vary greatly in terms of their taxonomic classification, their host and tissue specificities and mechanisms of parasitism. Different fungi cause soft rots of fruits, tubers, corms, bulbs, roots, stems, etc., and Table 9.1 lists some of the economically important rots of plants caused by fungi. Rots can occur in the field or in stored fruits, tubers and bulbs. In addition to causing a rotting of roots and stems of young plants, some fungi cause a rapid death and collapse of young seedlings, i.e. damping-off disease.

9.12.1 Pythium damping-off disease

Species of the genus *Pythium*, including *P. irregulare, P. aphanidermatum, P. debaryanum* and *P. ultimum* can cause root rot, seedling damping off and seed rot of many plants and soft rot of fleshy fruits that are in contact with the soil. Broadleaf and grass plant species are particularly susceptible to the disease. *Pythium ultimum* prevails in cooler to cold soils, while *P. irregulare* and *P. aphanidermatum* are adapted to higher soil temperatures. Like *Phytophthora*, *Pythium* species are soil-borne oomycetous fungi that produce asexual motile sporangia-derived zoospores (Plate 14(a)) and sexual oospores (Plate 14(b)), although some pathogenic isolates have no known sexual reproduction stage and most species are homothallic.

The symptoms caused by damping-off fungi such as *Pythium* species depend on the plant age and developmental stage. Seeds sown into infested soil can become infected and disintegrate. Post-germination seedling infections manifest as either a poor rate of emergence above the soil line (i.e. pre-emergence damping off), or the collapse of emerged seedlings (i.e. post-emergence damping off) (Plate 14(c)). *Pythium* species can attack and cause lesions of the stems or root rot of older plants that, if severe, can cause plant stunting, leaf yellowing, wilting and death. *Pythium* soft rots of fleshy vegetable organs manifest as woolly fungus growth on the surface (often referred to as 'cottony leak'), accompanied by internal soft rotting of the organ. These symptoms are also commonly caused by species of the genera *Rhizoctonia*, *Fusarium* and *Phytophthora*, other fungi and some bacteria.

Sporangia and thick-walled oospores constitute important soil-borne survival structures for *Pythium* species. Seed, root and stem infection (at the soil line) occurs when, under moist conditions, soil-borne mycelium or zoospore germ tubes come into contact with, and penetrate, the tissue (either directly or through cracks, wounds or natural openings). Exudates from seeds and roots can induce fungal spore germination, hyphal growth and penetration. The fungus then uses physical force and an enzymatic degradation of middle lamellae, cell walls and protoplasts to colonize the host, leading to pre-emergence death or to post-emergence damping off of seedlings. Once the tissue is colonized by mycelium, sporangia and oospores are produced inside or outside the host tissue and these return to the soil with decaying plant material, providing a new source of inoculum for future infections. The disease is spread through soil water and through the movement of infected plant debris.

Good resistance to *Pythium* damping off and associated diseases is virtually unknown amongst cultivars of many hosts. Cultural practices, including good sanitary practice, shallow seed planting, provision of adequate plant nutrition and maintaining well drained plots or beds, help prevent or reduce the risk of *Pythium* diseases. Because many plants are susceptible to these diseases, crop rotation may not prevent disease and inoculum build-up in soil. In warmer countries, pre-plant soil fumigation was commonly used in contained conditions (e.g. glasshouses) to kill soil-borne inoculum. Soil sterilization, and soil solarization, may reduce inoculum build-up. Systemic fungicides can be applied as a preventative seed dressing or as a water-soluble preventative or curative soil drench. However, as with many diseases, when fungicides are applied post-infection, efficacy in controlling disease will be reduced. The success of biological control agents in controlling *Pythium* diseases has been very variable, especially under field conditions. Bacterial that have shown potential to control *Pythium* diseases include species of *Pseudomonas*, *Burkholderia*, *Streptomyces* and *Bacillus*; fungal antagonists include species of *Trichoderma*, *Gliocladium*, and non-pathogenic *Pythium* and *Fusarium oxysporum* isolates.

9.13 Leaf and stem spots, anthracnose and scabs

Leaf and stem spots are characterized by the occurrence of localized lesions on either the host leaves or stems. Several fungi cause such foliar and stem diseases of cereals that manifest as localized spots (Table 9.1). Septoria leaf blotch (Plate 15) (and glume blotch) are considered amongst the most serious diseases of cereals, especially in maritime climates. Anthracnoses are diseases in which spots or sunken lesions with a slightly raised rim occur on the stems, foliage or fruits of plants; other symptoms of this disease may include dieback of twigs or branches and infected fruit may drop and rot (Table 9.1). Anthracnose diseases of curcurbits and common beans caused by *Colletotrichum* species cause significant yield losses worldwide; the latter is particularly serious in Africa, Central and South America and Asia (Plate 16). Scabs are characterized by localized lesions of scabby appearance on host fruit, leaves and/or tubers. Fungal scab is the most important disease of apples, reducing quality and size of infected fruit (causal organism = *Venturia inaequalis*).

9.13.1 Spot and blotch diseases caused by *Mycosphaerella* species

Mycosphaerella comprises an ascomycetous genus of fungi with anamorphs of various types e.g. *Cercospora*, *Septoria*, *Ascochyta*, *Ramularia* and *Didymella*. Associated disease names often incorporate the anamorph state. Between them, members of this genus, or their anamorph states, cause serious leaf spots of cereals, banana, pea and other vegetable crops. Economically important diseases caused by fungi from this genera include leaf spot diseases of banana caused by *Mycosphaerella* species, *Ramularia* blotch of barley caused by *Ramularia collocygni* (telomorph not known) and *Septoria* blotch of wheat caused by *Mycosphaerella graminicola* (anamorph: *Septoria tritici*).

Septoria blotch of wheat is amongst the most serious diseases of cereals, particularly in maritime climates. This fungus produces sexual ascospores and asexual conidia called pycnidiospores (produced in a spore case called a pycnidia). Disease symptoms include elongate oval lesions on leaves, running parallel to the leaf. These become water-soaked and brown, and a chlorotic halo may develop around the lesion. Black pycnidia develop on mature lesions and cirri may form on the lesions if the weather is dry for prolonged periods (Plate 15(b)). The fungus overwinters on seed, stubble, debris, and overwintering cereal crops. In the spring, it reproduces sexually to form ascospores that are wind-dispersed to wheat leaves. However, asexual pcynospores that are water-splashed to host leaves provide the major source of inoculum for disease development. Spore germ tubes directly penetrate the plant or enter via stomata, where they colonize host tissue and produce pycnidia. Moisture in the form of rain, dew or irrigation stimulates pycnidiospore release from conidia that are water-splashed to

leaves to cause new infections. Control of this disease is attempted by good sanitary practices such as removal of crop debris, crop rotation, using resistant wheat cultivars and chemical control. Many isolates of this fungus are resistance to certain fungicides. Systemic fungicides are used to control this disease, but recently resistance to the newer strobulurin fungicides has been reported amongst strains of the fungus. Some wheat cultivars show more resistance to the disease than others.

9.14 Rusts, smuts and powdery mildew diseases

Rusts, smuts and powdery mildew diseases are amongst the most common and devastating fungal diseases of plants. Control of rusts, smuts and powdery mildews is achieved by good cultural practices, using resistant hosts and fungicide application. Rust diseases have caused devastating losses on coffee and grain crops, but also of other field crops, pine, apple and ornamentals. There are thousands of rust fungi that attack different plants. They are basidiomycetes and most are obligate parasites. The most destructive diseases caused by rusts include stem rusts of wheat and other cereals caused by *Puccinia graminis* (Plate 17), coffee rust caused by *Hemileia vastatrix* and *Puccinia* rusts of field crops, vegetables and ornamentals caused by *Puccinia* species. Rust diseases appear as yellow to brown, white or black rusty spots; the rusty appearance results from epidermal rupture by emerging spore masses that often have a powdery appearance.

Smut diseases, also caused by basidiomycetes, were a serious problem prior to the development of contemporary fungicides. These fungi are not obligate parasites, i.e. they can be cultured on media, but in nature they exist almost exclusively as parasites. Most smut fungi attack and devastate the ovaries of cereal grains and grasses, turning seed to black powdery masses (e.g. covered smut of oats caused by *Ustilago hordei* and covered smut or 'bunt' of wheat caused by *Tilletia* species, Plate 18), but some attack leaves, stems or seeds. Smut diseases either destroy the affected tissue or replace it with black spores.

Powdery mildew diseases are amongst the most conspicuous plant diseases, affecting all kinds of plants. They are caused by obligate parasites that are members of the fungal family Erysiphaceae. Plate 19 depicts the symptoms of powdery mildew disease of wheat caused by *Blumeria* (= *Erysiphae*) *graminis*. Grasses and cereals are amongst the plants severely affected by this disease, as are other field crops, ornamentals such as roses and trees. Symptoms of powdery mildew disease include chlorotic or necrotic leaves, and stems and fruits covered with mycelium and fruiting bodies of the fungus, often giving a white 'woolly' appearance.

9.15 Global repercussions of fungal diseases of plants

The economic implications of plant disease are a reduction in crop or harvest value due to decreased quality and/or quantity of produce. Plant disease epidemics can directly or indirectly impact on the health of humans and animals. Direct impacts include mycotoxicosis associated with the consumption of mycotoxin-contaminated foods. Mycotoxins have also been investigated as potential biological warfare agents; but conversely, some mycotoxin derivatives have provided medicinal compounds beneficial to human health. Historic examples that clearly illustrate the far-reaching and indirect repercussions of fungal diseases of plants include ergot disease of rye and other cereals and the Irish potato famine.

Ergot is a disease of cereal heads caused by *Claviceps* species, including *C. purpurea* and *C. fusiformis*. In infected grain, *C. purpurea* produces ergotamine ergocristine alkaloid mycotoxins and *C. fusiformis* produces clavine alkaloid mycotoxins; these are responsible for gangrenous and convulsive ergotism diseases, respectively, of humans and animals. Gangrenous ergotism is characterized by a loss of extremities and convulsive ergotism normally manifests as a nervous dysfunction of the victim. In the Middle Ages these compounds caused many disease epidemics and outbreaks of ergotism have been documented throughout history. They were responsible for many thousands of deaths and social upheaval in Europe. In France in 800–900 AD the consumption of bread contaminated with toxins (and the ergot alkaloid derivative lysergic acid diethylamide (LSD) produced during the baking of contaminated wheat) caused what was termed *ignis sacer* (sacred fire) or St Anthony's fire disease of humans. This ergotism epidemic was so called because it was believed that a pilgrimage to St Anthony's shrine would alleviate the intense burning sensation associated with ergotism disease. It is theorized that women accused of witchcraft and sentenced to death in Europe and in the Salem trials of 1692 were suffering from ergotism. An outbreak of gangrenous ergotism occurred in Ethiopia in 1977–1978; convulsive ergotism occurred in areas of India in 1975. However, contemporary grain processing and cooking processes employed in the Western world eliminate or detoxify most ergot alkaloids present in grain. In contrast, ergot alkaloids have also yielded beneficial medicinal compounds; the commonly used derivative ergotamine is often prescribed for patients suffering from vascular headaches.

The advent and development of fungicides over the last century means that, today, the repercussions of fungal diseases are not as widespread as those of the Irish potato famine or ergotism in the Middle Ages. However, such compounds do not easily control some pathogens and others evolve resistance to fungicides. Also new pathogens and associated disease outbreaks are continuously emerging. Sudden oak death caused by *Phytophthora ramorum* is an example of such a disease. This has emerged over the last decade as a devastating disease of oak

and other forest trees in cooler wet climatic regions and has reached epidemic proportions along the Californian coast.

Plant pathogens and their toxic metabolites have been investigated as terrorism and military weapons and thus have raised public and security concerns, but the usefulness of such organisms as biological weapons is open to debate. Yellow rain caused thousands of deaths in South East Asia (1974–1981) and some suggest that T-2 toxin, which belongs to the trichothecene group of mycotoxins produced by some *Fusarium* species, was the agent of disease, but this is highly controversial. Strains of the non-trichothecene-producing *Fusarium* species *F. oxysporum* and the fungus *Pleospora papaveracea* that are natural pathogens of poppies are under investigation for their potential control of narcotic-yielding poppy fields to aid in the 'war on drugs'. However, the consequences of the deliberate release of such organisms or derivative compounds is highly controversial and a subject of world debate.

9.16 Conclusion

From this overview into plant pathology, it is very obvious that fungal diseases of plants have, and will in the future, put tremendous stress on world food production. From the wilts to the gall diseases, these fungi have evolved, and are continually evolving, diverse mechanisms for infecting and colonizing the world plant population. Plant pathology researchers have made a tremendous contribution to the science of understanding these diseases. The 'global economy' and changing cultural and breeding practices (e.g. organic farming and genetic engineering) are now placing further challenges on plant pathologists to determine how these virulent pathogens are moving around the world (gene flow), whether host or non-host disease resistance exists and how it can be introgressed into agronomically desirable crops and whether new environmentally friendly and durable chemical and biological disease control methods can be developed. Both traditional and novel molecular biology techniques are being adopted to answer these questions in order to safeguard the world plant population.

9.17 Acknowledgements

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9.18 Further reading

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9.19 Revision questions

Q 9.1 Define the terms parasitism, symbiosis and saprophytism.

Q 9.2 What is the disease triangle?

Q 9.3 Describe a generalized disease cycle.

Q 9.4 Describe how enzymes produced by fungi are critical to pathogenesis.

Q 9.5 Define the two types of fungal toxin.

Q 9.6 Define protectant, systemic and eradactive in relation to fungicides.

Q 9.7 Give an example of the use of direct biological control for the control of fungal pathogens.

Q 9.8 What plants are affected by Verticillium wilts?

Q 9.9 List some of the global impacts of fungal diseases of plants.

Answers to Revision Questions

Chapter 1

A 1.1 Principal macromolecular components of both yeast and fungal cells: glucans, chitin and mannoproteins arranged in microfibrillar network. Major difference between yeasts and fungi: the predominance of chitin in the latter. Major physiological roles: cell structure stability, stress protection and cell–cell interactions.

A 1.2 Main nutrients are sugars for carbon and energy; ammonium or amino acids for protein synthesis; oxygen for respiration and fatty acid/sterol synthesis; minerals for ionic balance and enzyme activity; vitamins for coenzymes/growth factors. Nutrients transported, depending on the type of nutrient, by active (energy-mediated) transport or by diffusion (free, facilitated or channels).

A 1.3 (a) Oxygen used as terminal electron acceptor in respiratory metabolism of glucose (via glycolysis, then mitochondrial oxidative phosphorylation) to water and carbon dioxide. (b) Fermentative metabolism of glucose by glycolytic enzymes in the cytoplasm to ethanol and carbon dioxide. Much more energy in the form of ATP generated by (a) compared with (b).

A 1.4 Temperature, pH, oxygen and osmotic pressure. Concept of minimum, optimum and maximum ranges for these factors and some typical values for fungal growth.

A 1.5 Diagrammatic description of budding cycle in yeast cells, typified by *Saccharomyces cerevisiae*. Description of hyphal elongation, septum formation in filamentous fungi.

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A 1.6 Logarithmic doubling, for a limited time period (and after recovery from a lag phase), of cells as nutrients are plentiful and if physical growth conditions are conducive. Calculations of doubling time given on p. 31.

Chapter 2

A 2.1 Fungi are very amenable to molecular/genetic analysis, as they grow very quickly, are easy to culture, many have a haploid genome which aids mutagenesis studies, many genomes have been sequenced, and advanced tools such as a genome-wide knock-out set in *S. cerevisiae* are available. Fungi are simple eukaryotic cells that exhibit many properties and regulatory mechanisms present in human cells.

A 2.2 Yeast cells grow vegetatively by budding, and daughter cells completely separate. Filamentous fungi grow vegetatively through highly polarized cells called hyphae. Hyphae do not separate into different cells, but lay down septum cross-walls, which separate the hypha into distinct compartments.

A 2.3 An ascus of *S. cerevisiae* contains four ascospores which can be isolated. The individual products of meiosis can therefore be tested. In *N. crassa*, the four products of meiosis go through an additional round of mitosis, creating eight ascospores. The asci are linear, which allows ordered arrangement of the ascospores and mapping of genes in relation to the centromere.

A 2.4 Metabolic mutants were identified in *N. crassa*. Conidia were irradiated to induce mutagenesis, then maintained on rich media to allow growth. Conidia were then tested for their ability to grow on minimal media. Growth could be restored on minimal media supplemented with a specific amino acid, allowing the identification of individual genes responsible for particular metabolic pathways.

A 2.5 Temperature sensitivity was used in a screen by Morris to identify cell cycle and nuclear distribution mutants in *A. nidulans*. Drug resistance was used to identify tubulin genes in this organism, as well. Sensitivity to mating pheremone was used to identify components of the mating pathway in *S. cerevisiae*.

A 2.6 *C. albicans* is diploid, and does not undergo meiosis.

A 2.7 Tetratypes are rare. Tetratypes require that the two genes are on the same chromosome and cross-over takes place, or there is cross-over between at least one gene and the centromere. Both genes are close to the centromere, and on different chromosomes. This would not change if the starting parents were the different genotype indicated.

A 2.8 *N. crassa* mating loci are called idiomorphs because the sequences vary significantly between opposite mating types, whereas the mating types are

referred to as alleles in *S. cerevisiae*. In *N. crassa* the mating loci also regulate heterokaryon compatibility, and mating type switching does not occur. In *C. cinereus*, there are more than 12 000 mating types as opposed to two in *S. cerevisiae*.

A 2.9 Parasexual genetic analysis involves recombination in the absence of meiosis, or mitotic crossing-over. This has been used in *A. nidulans* to help map genes to chromosomes. In *C. albicans*, meiosis does not occur but tetraploid nuclei formed through mating can break down into diploid products, which demonstrate different genotypes from the parents. Diploids form from tetraploids through chromosome loss, indicating that mitotic crossing-over gave rise to the progeny.

A 2.10 Plasmids in *S. cerevisiae* can be maintained as extrachromosomal elements, as they contain an autonomous replication sequence (ARS) and centromere sequence (CEN). These allow the plasmids to be stable and to segregate. Genes can be introduced and expressed without integrating into the genome, and plamid loss can be induced.

Chapter 3

A 3.1 Because not only was it the first eukaryotic organism to have its genome sequenced, but also its homologous recombination system allowed for the systematic deletion of each ORF in search of phenotypes; microarray technology allowed its global mRNA profiles to be identified and two-hybrid technology looked at all possible protein–protein interactions. Moreover, *S. cerevisiae* has now become a central player in the development of systems biology.

A 3.2 Pre-genomic research was characterized by hypothesis-driven sequential experiments; post-genomic research is driven by the massively parallel analysis of biological information, followed by pattern recognition within datasets.

A 3.3 This is the name given to a cross-disciplinary approach to developing computer models of how molecules interact to generate biological phenomena. It includes contributions from biology, chemistry, physics, mathematics, computer science and engineering.

A 3.4 This is a sequence of DNA that starts with a methionine codon and runs for another 99 codons, without hitting a nonsense one.

A 3.5 Approximately 5000 new ORFs were discovered. Approximately 3000 ORFs were annotated with functions. A further 1000 have had functions attributed to them since then.

A 3.6 That *S. cerevisiae* underwent *genome* duplication, *S. pombe* underwent many *gene* duplications and that both have active transposable elements. On

the other hand, there is no evidence of genome or multiple gene duplication in *N. crassa*. Moreover, the latter also displays a paucity of transposable elements.

A 3.7 *In silico analysis* uses BLAST searches of previously annotated DNA sequences to provide insight into an ORF's function depending on how well matched the new sequence is to ones already in the databases. *Reverse genetics* associates phenotypes with deliberately disrupted known ORFs. *Identification by association* requires a comparative analysis of genomes from closely and distantly related organisms, thereby identifying genes that are held in common by biologically related species. e.g. a gene that is found in yeasts but not in filamentous fungi probably encodes information that defines some specific aspect of yeast biology and vice versa.

A 3.8 DeRisi *et al*. PCR-amplified each of the 6400 distinct ORFs described in the first edition of the yeast genome and printed these DNA molecules at unique addresses on glass slides using a simple robotic printing device. This was then used simultaneously to explore the expression profile of mRNA as yeast cells underwent a diauxic shift from fermentative growth to aerobic respiration and to explore gene expression patterns in regulatory mutants of yeast. Red fluorescently-labelled cDNA was synthesized from cells grown under different culture conditions. Green fluorescent cDNA was also prepared using mRNA from control cells. The expression level of each gene was measured by hybridizing fluorescently labelled cDNA to the microarray probe, visualizing the fluorescence pattern using a confocal microscope and using a computer to analyse the image of the relative intensity of the spots. Yellow spots indicate that the experimental and control cells express that particular ORF equally. Red colour indicates gene expression increased relative to the reference, while green colour indicates gene expression decreased relative to the reference. The analysis revealed that 28 per cent of all yeast genes underwent a significant alteration in gene expression level as a result of a diauxic shift and identified groups of genes whose pattern of expression changed in association with one another. It also detected coordinately regulated genes in the regulatory mutants.

A 3.9 *BLAST* (Basic Local Alignment Sequence Tool): an algorithm that searches a sequence database for sequences that are similar to the query sequence. There are several variations for searching nucleotide or protein databases using nucleotide or protein query sequences. *Complementary DNA library*: a collection of clones, each containing a cDNA derived from the RNAs isolated from a specific tissue or cell and inserted into a suitable cloning vector. *Expressed sequence tags*: short cDNA sequences that are derived from sequencing of all the mRNAs present in a cell. ESTs represent the expression profile of the cell at the time point of RNA isolation. *SAGE* (serial analysis of gene expression): extremely short ESTs that are linked together as DNA chimeras consisting of 15-base-pair sequences from 40 different mRNAs. The sequence analysis of thousands of these 40×15 bp chimeras permits a quantitative

estimation of the mRNAs in the original sample. *Sequence alignment*: a linear comparison of nucleotide or protein (amino acid) sequences. Alignments are the basis of sequence analysis methods and are used to identify conserved regions (motifs).

A 3.10 The challenges facing proteomics in defining 4D biology include establishing the amino-acid sequence and 3D structure of every protein encoded by the genome. It also requires information on how the location and concentration of each protein change with alterations in the cell. It also needs to establish when and where proteins are post-translationally modified, and finally how proteins interact with other proteins in different contexts.

A 3.11 Fusion proteins are made by using recombinant DNA techniques to splice, in frame with a yeast promoter, the DNA sequence for each yeast gene to the DNA sequence encoding the other member of the fusion protein. Fusion proteins find two major uses in post-genomic yeast analysis. (a) Fluorescent protein fusions are used to visualize where each yeast protein is located in the cell. (b) The two-hybrid system exploits the fact that transcription factors consist of two separable protein domains: a DNA-binding domain and an activation domain. If the DNA-binding domain is fused to one yeast protein and the appropriate activation domain is fused to another yeast protein then the two domains will only be close enough to drive expression from a reporter gene if the two yeast proteins interact closely in the cell. If yeast protein X is fused to a DNA-binding domain protein (DBD) and yeast protein Y is fused to the appropriate activation domain (AD), specific interaction of proteins X and Y brings the DBD and AD together, and when the DBD binds to the promoter the AD can drive reporter gene transcription. If X and Y do not interact, then the DNA-binding domain can still bind to the promoter but, in the absence of the activation domain, cannot activate expression.

A 3.12 In *step 1* of the systems approach all available information is used to define an initial model of the phenomenon. In *step 2* each known component is systematically perturbed, yielding numerous separate cellular conditions. The global cellular response to *each perturbation* is detected and quantified using array technology. Also, in at least some of these cases, large-scale protein expression analysis is performed. In *step 3* all of this new information is integrated with the initially defined model and also with the information currently available on the global network of protein–protein and protein–DNA interactions in yeast. In *step 4* the researchers formulated new hypotheses to explain the observations not predicted by the model, and then designed and executed additional perturbation experiments to test the new 'improved' model. This is then re-iterated until all of the observations can be accommodated within one coherent model.

Chapter 4

A 4.1 Solid fermentation is used in the production of edible mushrooms and involves growing mycelium over a solid substrate. Batch fermentation is employed in the production of beers and wines and involves growing yeast in a liquid medium derived from barley or grapes, respectively. Fed batch is used for the production of baker's yeast and involves adding substrate to the fermentation at pre-determined times to increase the cell density and uses the cells from the end of one fermentation to start the next fermentation. Continuous fermentation is used in the manufacture of Quorn mycoprotein and involves keeping the fungus at a steady state to maximize the production of biomass.

A 4.2 Cells can be entrapped within a membrane, attached to a solid substrate, entrapped in a bead composed of alginate, for example, or induced to flocculate to give higher densities than might normally be expected. Immobilization ensures that a high cell density is maintained and removes growth inhibition due to the production of a toxic metabolite. The main disadvantage of immobilization is that fungal cell viability decreases over time and that the immobilization system may degrade with continual usage.

A 4.3 Productivity can be affected by a variety of factors. The nature of the fermentation system is a major consideration since a particular system may be good for producing one product but bad for another. For example, batch fermentation is used for ethanol production but not usually for antibiotic production by fungi. The physical nature of the fungal cell is important – yeast cells can be agitated with spargers (paddles) but the fungal mycelium will be disrupted if spargers are employed. The level of aeration will influence productivity particularly when ethanol is being produced. Too high a concentration of sugar can adversely affect productivity.

A 4.4 Novel means of genetically manipulating yeast have allowed the creation of brewing strains with altered characteristics. For example, yeasts have been produced that (a) do not produce the organoleptic diacetyl, (b) contain genes for amylase and glucanse production and (c) flocculate at the end of the fermentation. Although not used commercially, genetically modified brewing strains should allow beers to be produced faster, cheaper and with new flavours/aromas.

A 4.5 Quorn mycoprotein is produced under continuous conditions in an airlift fermenter. Fungal mycelium is fed a medium composed of glucose, biotin and mineral salts at a constant rate. Culture is harvested at the same rate as new medium is added to the fermenter and thermally treated to reduce the RNA content.

A 4.6 The synthesis of antibiotics by fungi can be affected by the nature of the fermentation system, the level of nitrogen or phosphate in the culture medium and feed-back inhibition where the antibiotic inhibits its own synthesis.

A 4.7 Historically fungi were cultivated under solid fermentation conditions for the isolation of enzymes. More recently submerged, fed-batch and continuous systems have been utilized.

A 4.8 The compost is spawned with a culture of *Agaricus bisporus* (edible mushroom) grown on sterilized cereal grains and cultured at 25–28 °C and high humidity for 14 days to allow the mushroom mycelium to colonize the compost. After this period, the compost is 'cased' with a layer of neutralized peat, which has the effect of stimulating the formation of large primordia, which will later develop into mushrooms.

A 4.9 'Rare mating' has been used with brewing strains of *S. cerevisiae*. In this process high numbers (10^8) of complimentary non-mating cultures of yeast are mixed, and occasionally some spontaneous mating occurs to give hybrids that may express improved fermentation characteristics.

A 4.10 Yeast are good candidates for the expression of foreign genes because they are easy to culture, are generally regarded as safe, are eukaryotic organisms and contain their own plasmids. From a molecular biology point of view efficient transformation systems have been developed and yeast splice introns in animal genes.

Chapter 5

A 5.1 Examples of primary metabolites: enzymes, industrial alcohol, organic acids, fats, polymers. Examples of secondary metabolites: antibiotics, cholesterol lowering agents, immunosuppresants, hormones.

A 5.2 Peptidoglycan.

A 5.3 It inhibits the production of interleukin-2 by T-lymphocytes.

A 5.4 They act as competitive inhibitors of the enzyme HMG-CoA reductase, which is a key enzyme in the cholesterol biosynthesis pathway.

A 5.5 Table 5.3 lists numerous examples of enzymes of economic importance, all of which are correct.

A 5.6 *Cephalosporium acremonium* is the main producer organism for cephalosporins.

A 5.7 The nutritional essentiality of selenium was established on the basis of its interaction with vitamin E. Beneficial effects of dietary selenium were first seen in vitamin E-deficient rats and selenium was shown to prevent some of the symptoms of vitamin E deficiency. High-selenium yeast has gained increased significance as a nutritional supplement due to its enhanced bioavailability over inorganic selenium (sodium selinite). Inorganic selenium is poorly absorbed and

metabolized by animals. Adequate intake of selenium is needed for immunocompetence; deficiency results in insufficient cell humoral immune response in both humans and animals.

Chapter 6

A 6.1 *Recombinant DNA* simply requires the cutting and joining of DNA molecules from two different sources. However, the production of *recombinant proteins* requires DNA engineering, and also the information has to be in the correct context to be transcribed and then translated. This means the careful insertion of the DNA downstream of an appropriate promoter so that it is in the correct reading frame. It often also requires a terminator sequence to be present in the vector. Once transcribed, the mRNA must carry the appropriate translation signals. Finally, the protein needs to fold properly (and often requires further processing by addition of sugars etc.) before it has the correct structure and hence biological function.

A 6.2 A *cDNA* sequence is derived by reverse transcription of the heterologous mRNA It therefore lacks introns and regulatory sequences and is therefore easier to have expressed in the majority of heterologous expression systems.

A 6.3 A backbone of bacterial plasmid DNA carrying a bacterial origin of replication and a selectable marker for use in *E. coli*. A selectable marker for the intended fungal host. A strong promoter to drive the production of the heterologous mRNA. Appropriate DNA sequences to ensure efficient termination of transcription and polyadenylation of the mRNA. Appropriate sequences to ensure the correct initiation and termination of translation.

A 6.4 Enzymatically removing the cell walls and exposing the resulting sphaeroplasts to the DNA in the presence of calcium ions and polyethylene glycol. Electroporation of yeast cells and fungal sphaeroplasts. Transformation of yeast cells by treating them with alkali cations (usually lithium) in a procedure analogous to *E. coli* transformation.

A 6.5 *Saccharomyces cerevisiae* grows rapidly by cell division. It has its own autonomously replicating plasmid. It can be transformed as intact cells. It forms discrete colonies on simple defined media. It can carry out post-translational modifications of expressed proteins. It secretes a small number of proteins into the growth medium, which can be exploited to simplify the purification of heterologous proteins. It has a long safe history of use in commercial fermentation processes. It does not produce pyrogens or endotoxins.

A 6.6 *Comparison*. Both have a backbone of bacterial plasmid DNA carrying a bacterial origin of replication and a selectable marker for use in *E. coli*. Both carry appropriate promoter and terminator sequences. Both carry appropriate selectable markers for use in their respective host cells.

Contrast. The yeast plasmid is an autonomously replicating one maintained at high copy number in the yeast cells. The *Pichia* vector cannot replicate autonomously in the fungal cells but integrates into the AOX1 locus by homologous replication. Also the copy number will be very low – unlike the yeast one.

A 6.7 The copy number of the expression vector. The strength of the promoter. The presence of a terminator. The presence of appropriate translation signals in the mRNA. Low-level protease activity. The production of an authentic protein product (glycosylation and/or processing by proteases).

A 6.8 Although the viral protein was expressed it was not glycosylated. Furthermore, the host cells failed to produce the 22 nm phospholipid–protein particles. The *E. coli* recombinant protein therefore failed to elicit an appropriate immune response in animals.

A 6.9 *Comparison*. Both have a backbone of bacterial plasmid DNA carrying a bacterial origin of replication and a selectable marker for use in *E. coli*. Both are autonomously replicating plasmids.

Contrast. The *Trp1* selectable marker in the proof of principle plasmid was replaced by the poorly expressed *Leu-2d* gene. This caused an increase in plasmid copy number. A terminator sequence was inserted after the gene in the scale-up plasmid to ensure that stable mRNAs were transcribed. The promoter was changed from *ADH1* in the proof of principle plasmid to the more powerful *GPDH* one in the scale-up version.

A 6.10 Use homologous recombination to insert a reporter gene construct into the yeast chromosome. This consists of a selectable marker (*URA1*) and a disabled *cyc1* promoter, containing oestrogen response elements, fused to a *LACZ* gene. Arrange to constitutively express the receptor protein from an autonomously replicating expression vector. In the presence of oestrogen the receptor protein binds to the oestrogen response elements in the promoter of the *LACZ* gene. The level of β-galactosidase activity is therefore a measure of how successful the receptor–oestrogen interaction is in terms of ERE-mediated gene activation. It can therefore be used to assay the effect of molecules that interfere with/accentuate the hormone–receptor interaction.

A 6.11 Under normal conditions when the mating pheromone α-factor binds to the α-factor receptor in Mat a cells it triggers a pathway that activates a number of genes, e.g. *FUS1*, through a promoter element and causes the cells to arrest their growth in the G1 phase of the cell cycle through *Far1*.

The yeast mating pathway was re-engineered so that the α-factor receptor is replaced by a heterologous human receptor protein (SST_2). Binding of the appropriate human ligand (somatostatin) results in activation of the mating pathway cascade. However, by deleting *Far1* the cells fail to arrest their cell cycle, and by fusing the *HIS3* gene to the *FUS1* promoter the cascade triggers the expres-

sion of *HIS3*. The ligand–receptor interactions can therefore be assayed by the number of cells with a *HIS3*+ phenotype.

A 6.12 The two-hybrid system exploits the fact that transcription factors consist of two separable protein domains: a DNA-binding domain and an activation domain. If two proteins interact closely in the cell, then if one is attached to a DNA-binding domain and the other protein is attached to the appropriate activation domain the two domains will be close enough to drive expression. Therefore proteins are genetically engineered so that protein X is fused to a DNA-binding domain protein (DBD) and expressed from one vector whereas protein Y is fused to the appropriate activation domain (AD) which is expressed from vector. Specific interaction of proteins X and Y brings the DBD and AD together and when the DBD binds to the promoter the AD can drive reporter gene transcription. If X and Y do not interact then the DNA-binding domain can still bind to the promoter but, in the absence of the activation domain, cannot activate expression.

Chapter 7

A 7.1 Ringworm (tinea) and athlete's foot (tinea pedis). These infections are caused by dermatophytes, such as *Trichophyton* spp. and *Microsporon* spp.

A 7.2 *Candida albicans*, *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*.

A 7.3 HIV infection, solid organ and bone marrow transplantation, antineoplastic therapy, premature birth, catheterization, broad-spectrum antibiotic and steroid treatment, prior colonization with *Candida*.

A 7.4 (a) The ability to grow in yeast and hyphal form (i.e. dimorphism), (b) phenotypic switching, (c) the ability to produce adhesins that allow *Candida* cells to attach to host tissues and (d) the ability to produce extracellular hydrolytic enzymes, such as aspartyl proteinases and phospholipases.

A 7.5 Invasive aspergillosis, aspergilloma and allergic bronchopulmonary aspergillosis.

A 7.6 *Pneumocystis jiroveci*, which is the causative agent of pneumocystis pneumonia (PCP).

A 7.7 Aflatoxin B_1 is the most clinically important form of this family of mycotoxins. It is believed to be carcinogenic; in particular, ingestion of food contaminated with aflatoxin B_1 may be a risk factor for hepatic cancer.

Chapter 8

A 8.1 Loss of intracellular K^+.

A 8.2 Amphotericin B.

A 8.3 Nystatin.

A 8.4 Amphotericin B.

A 8.5 It is converted to 5-fluorouracil, which can be incorporated into fungal RNA or serves as an inhibitor of thymidylate synthetase.

A 8.6 Interfere with the biosynthesis of ergosterol in susceptible fungi.

A 8.7 Clotrimazole.

A 8.8 Fluconazole and amphotericin B.

A 8.9 Itraconazole.

A 8.10 Terbinafine.

A 8.11 Naftifine.

A 8.12 Micafungin.

A 8.13 Nikkomycin and polyoxins.

Chapter 9

A 9.1 *Parasitism* occurs where one species lives off another, as distinct from *symbiosis*, where different species live in harmony with each other and the relationship is mutually beneficial, or *saprophytism*, where organisms grow on dead organic matter.

A 9.2 The disease triangle may be defined as the relationship between a phytopathogen and plant and the development of disease, which is influenced by the nature of the pathogen and host, and the prevailing environmental conditions.

A 9.3 Inoculum is produced and disseminated, and on reaching its target host plant tissue (inoculation) it penetrates the host. The type and mode of production of inoculum (e.g. sexual and asexual spores, resting spores, mycelium) and the method of dissemination (e.g. wind, water, insect) depends on the particular pathogen. For many important plant pathogens, a sexual stage has not been identified. Penetration is through wounds or natural plant pores (e.g. stomata), and some fungi produce specialized penetration structures called appressoria (singular = appressorium). Having penetrated its host, the fungus then grows within plant tissue (infection). The incubation period defines the period between

inoculation and infection. As it grows within the host, it utilizes the plant's cellular resources as a nutrient source and the damage inflicted on the plant is manifested as disease symptoms. The latent period defines the period between infection and symptom development. The pathogen forms survival structures such as spores that are then disseminated. During the plant pathogenesis, some diseases only involve one such disease cycle (primary cycle), while others have the potential to do more damage as they involve secondary or multiple cycles of disease.

A 9.4 Fungi produce a range of enzymes that facilitate host plant infection and colonization by degrading the cellular and intercellular constituents of plants (certain fungal pathogens also produce non-enzymatic proteins that inhibit the activity of plant enzymes involved in the host defence response).

A 9.5 Fungal toxins may be host or non-host specific. Host-specific toxins are usually required for pathogenicity, but are only toxic to the host plants of a pathogen, and show no or low toxicity to other plants. In contrast, non-host-specific toxins affect a wide range of host plants, and may act as virulence factors increasing the extent of the disease, but are not essential for disease development.

A 9.6 *Protectant*: do not penetrate the plant, but affect pathogen viability and germination on the surface of the host plant. Such fungicides must adhere to the plant surface and resist weathering and are most effective when applied as a preventative measure, i.e. before plant inoculation with the fungus. *Systemic*: can act on the plant surface and be translocated throughout the plant vascular system to kill the fungus. Systemic fungicides may also exhibit translaminar movement within the leaves. *Eradactive*: they are applied post-infection and act on contact by killing the organism or by preventing its further growth and reproduction.

A 9.7 The biological control agent suppresses the pathogen by one or more of the following means: hyphal interference or secretion of antifungal compounds (e.g. antibiotics) or enzymes. The fungus *Trichoderma harzianum* is used for the biocontrol of various fungi, which it achieves by means of hyphal interference and the secretion of hydrolytic enzymes that attack the causal fungi.

A 9.8 Vegetables, flowers, field crops, fruit trees, roses and forestry trees.

A 9.9 Famine (Irish potato famine 1845–1849), ergotism (Ethiopia in 1977–1978), disease due to mycotoxins, potential use of fungal toxins as bioterrorism agents.

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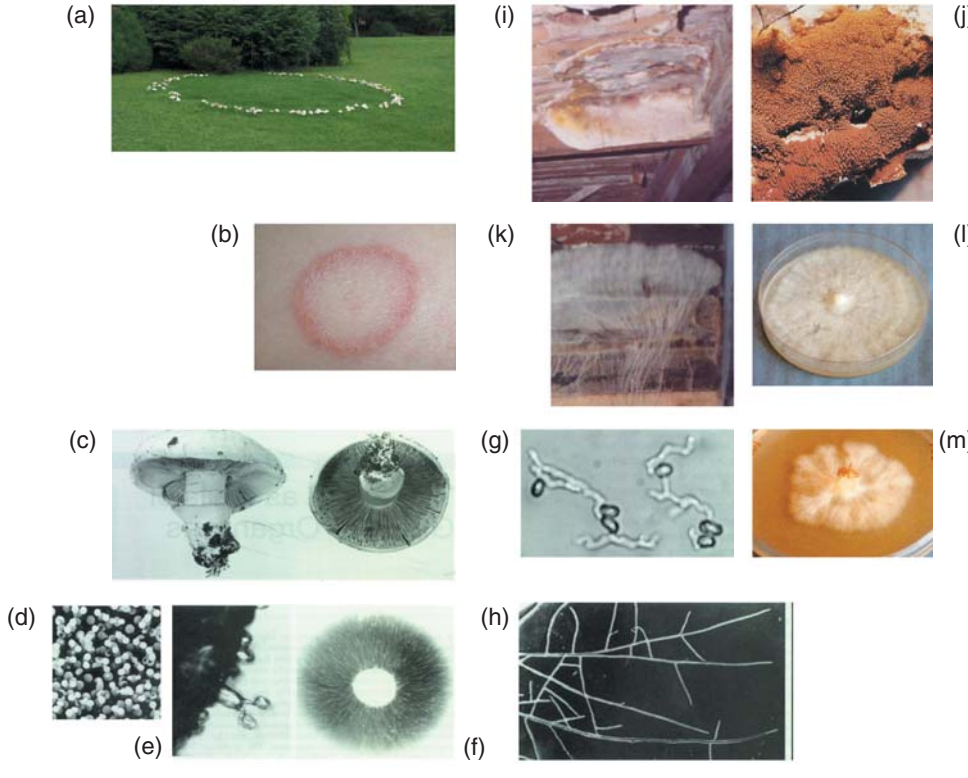

Plate 1 Radiating circular pattern of (a) *Chlorophyllum molybdites* fruit-bodies forming a fairy ring in grassland and (b) *Tinea corporis* infection of the skin. (c) Fruiting bodies of *Agaricus bitorquis*, (d) scanning electron micrograph of *Agaricus bisporus* gill surface (P.T. Atkey), (e) light micrograph of basidium bearing two spores (T.J. Elliott), (f) spore print from underside of fruit-body (M.P. Callen), (g) germ-tubes emerging from spores (T.J. Elliott; published by John Wiley & Sons, Ltd), (h) branching hyphae growing on an agar plate (T.J. Elliott) (all from Carlile, Watkinson and Gooday (2001) Academic Press). The dry-rot fungus *Serpula lacrymans* (i) colony decaying timbers in a wall void and forming a red-brown fruiting structure, (j) close-up of underside of a basidiocarp (from Ingold and Hudson, published by Chapman & Hall, Ltd), (k) exploratory fan-shaped mycelium with connected rhizomorphs, (l) fast- and (m) slow-growing colonies growing on agar media.

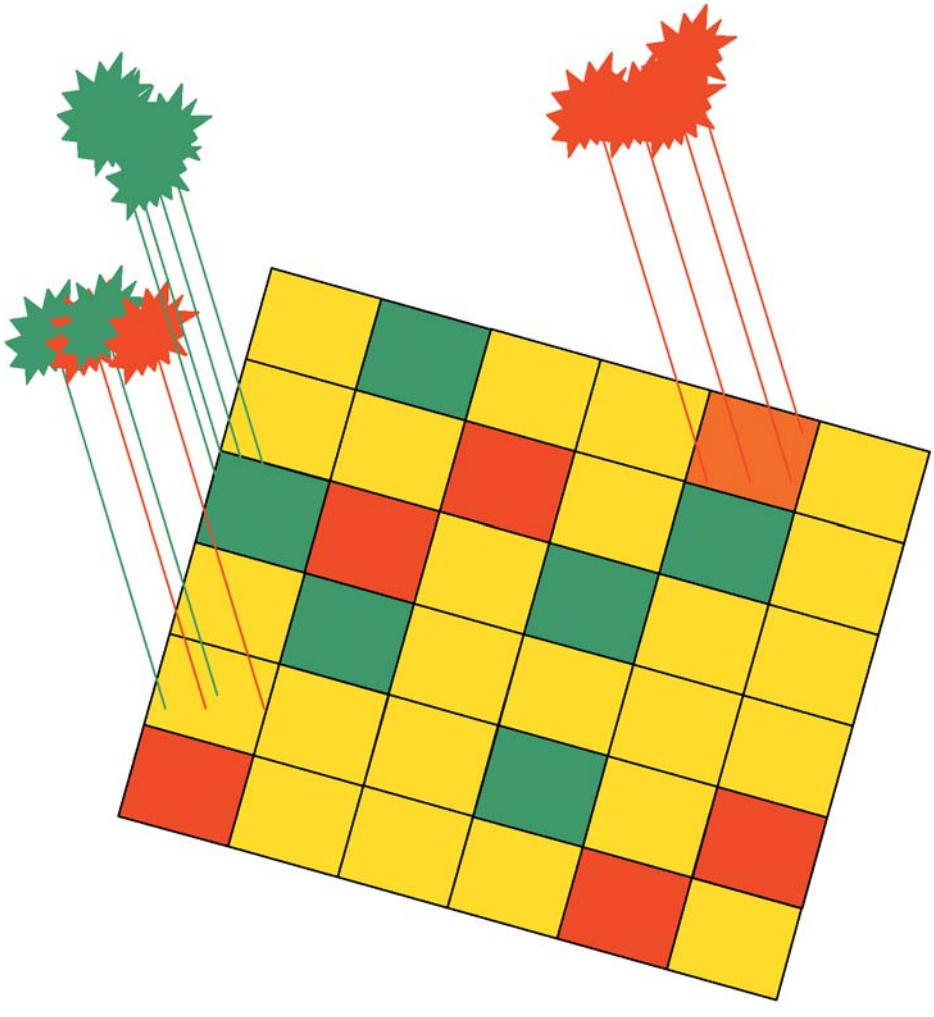

Plate 2 Two-colour microarray technology. 6400 distinct ORFs are arrayed on the slide (only 36 are shown here). Two separate cDNA samples, one from yeast growing in glucose at the beginning of the fermentation labeled with a green fluorescent dye and the other from later in the fermentation labelled with the red dye. Red colour indicates gene expression increased relative to the reference, green colour indicates gene expression decreased relative to the reference and yellow colour indicates no change in expression level. Three ORFs are shown in detail here.

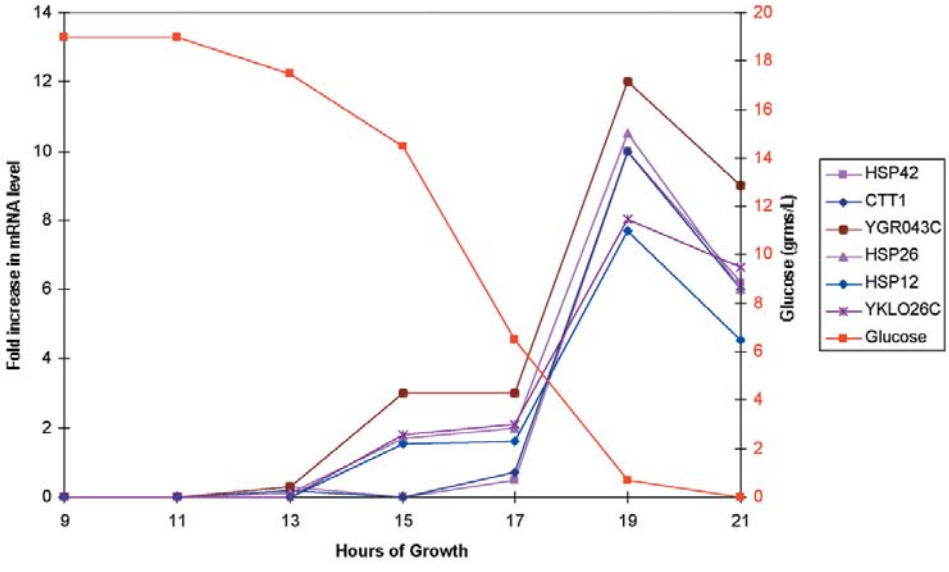

Plate 3 Pattern interpretation of microarray (after deRisi *et al.*, 1997). This shows how the expression levels of genes known to be stress-induced via STREs in their promoters were identical to one another. (The promoter sequences of other genes with the same profile but not previously identified as stress-inducible were then examined – many contain one or more recognizable STRE sites.)

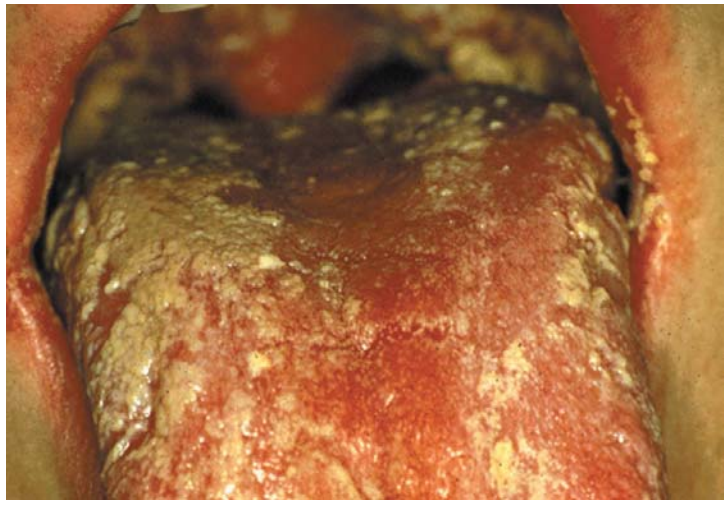

Plate 4 A photograph of the oral cavity of a patient with pseudomembranous oral candidosis (thrush).

Plate 5 A photograph of a CHROMagar Candida™ plate on which a variety of *Candida* species have been cultured. Note that most species can be differentiated on the basis of the colour of the colonies produced.

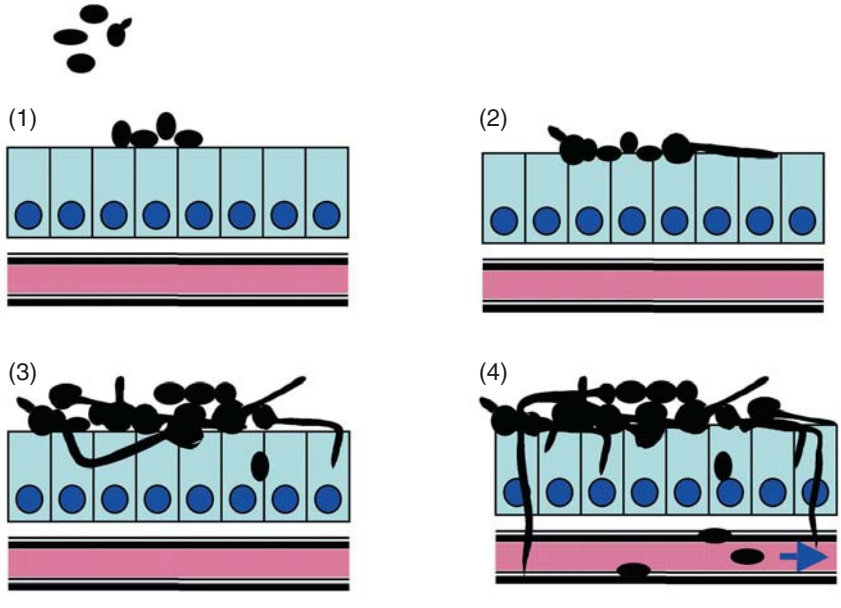

Plate 6 A schematic diagram indicating the stages of candidal infection.

Plate 7 Variation in the head blight susceptibility of two barley cultivars to Fusarium head blight disease. Both heads were inoculated with *Fusarium culmorum*, but only the cultivar on the left shows disease symptoms (premature bleaching of head spikelets).

(a)

(b)

Plate 8 Effect of deoxynivalenol, a non-host-specific fungal trichothecene toxin, on the germination of wheat seedlings. Seeds germinated on toxin solution (a) show reduced root and coleoptile growth compared with those germinated on water (b).

Plate 9 Clubroot of brassicas caused by *Plasmodiophora brassicae*. Roots swell and develop tumours due to increased hormone (e.g. cytokinin) production.

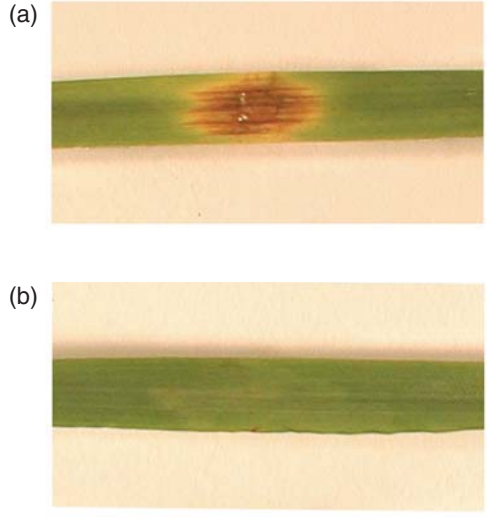

Plate 10 Biological control of net blotch disease of barley (caused by *Perenophora teres*) by a bacteria (*Bacillus* spp.) originating from a cereal field. The blotch disease symptom severity caused by this pathogen on inoculated leaves in the absence (a) and presence (b) of the biocontrol agent.

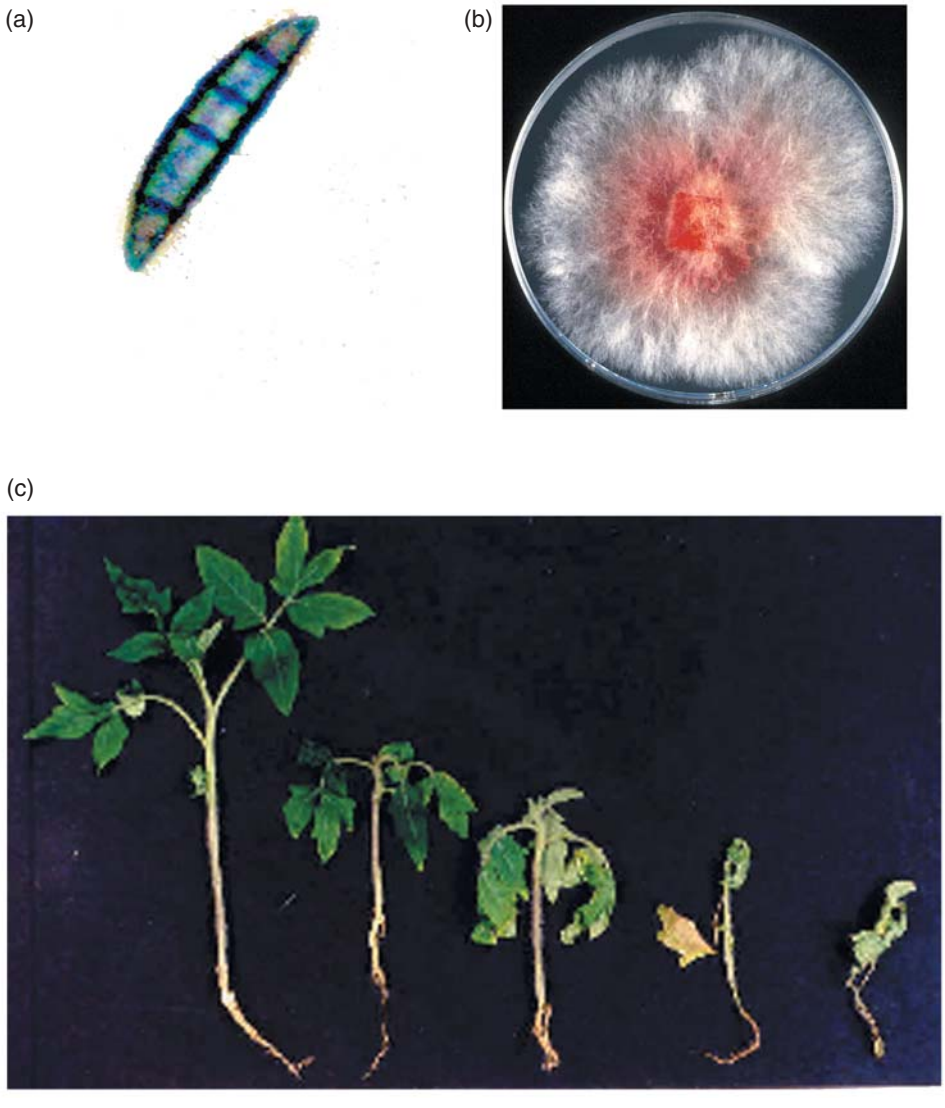

Plate 11 Fusarium wilt. Microscopic analysis of typical asexual macroconidia (a) and mycelial growth on potato dextrose agar (b) of *Fusarium* species (*Fusarium oxysporum* also produces microconidia and chlamydospores). (c) *Fusarium oxysporum* f. sp. *lycopersici* wilt of tomato: symptoms range from healthy (left) to severe wilting and stunting (right) (courtesy Dr Antonio Di Pietro, Cordoba, Spain: Di Petro *et al.*, 2003).

Plate 12 Yellowing/browning of leaves of an elm branch due to Dutch elm disease. The entire tree subsequently wilted and died.

(a)

(b)

(c)

(d)

Plate 13 *Phytophthora infestans* late blight of potatoes. Microscopic analysis of asexual sporangia containing zoospores (a) and sexual oospore (b). Disease symptoms: water-soaked dark lesions on leaves (c) and diseased tubers exhibit brown/black blotches on their surface, and internally, they exhibit water-soaked dark-brown rotted tissue (d).

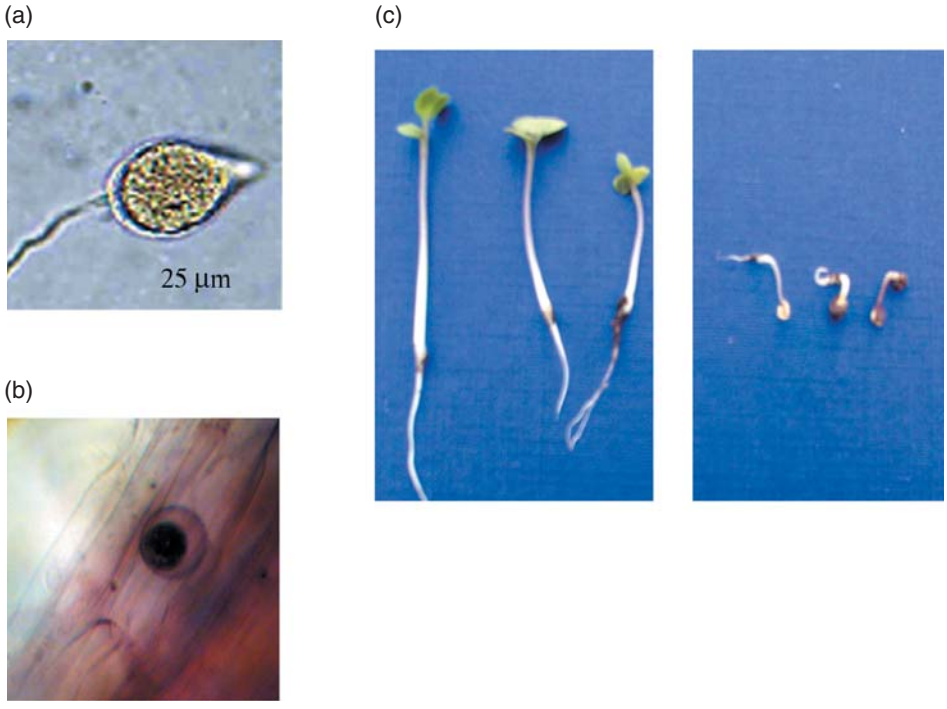

Plate 14 *Pythium* damping-off disease. (a) Asexual sporangia containing zoospores; (b) sexually-produced oospore formed on the surface of a tomato seedling; (c) seedlings of cress that are healthy (left) and others exhibiting damping-off symptoms (right).

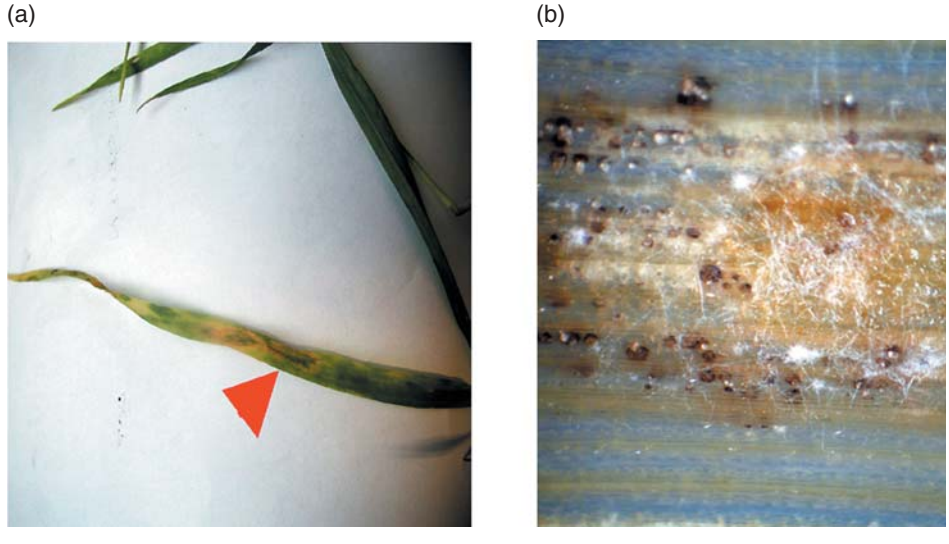

Plate 15 Septoria leaf blotch of wheat caused by *Mycosphaerella graminicola*. (a) Oval disease lesions (surrounded by a chlorotic halo) on an infected leaf, running parallel to the leaf blade; (b) black pycnidia develop on mature lesions and cirri may form on the lesions if the weather is dry for prolonged periods.

Plate 16 Sunken lesions on broad-bean pod due to anthracnose disease caused by *Colletotrichum lindemuthianum*.

Plate 17 Black stem rust of mature wheat stems caused by *Puccinia graminis* f. sp. *tritici*. The three stems possess black pustules of teliospores; these pustules arise from red urediospore pustules that erupt through the stem epidermis earlier in the growing season.

Plate 18 Smut diseases of cereals. Covered smut of oats caused by *Ustilago hordei* (a) and covered smut or 'bunt' of wheat caused by *Tilletia* species (b). Grain are replaced with black spore masses and hence destroyed.

Plate 19 Powdery mildew disease of wheat caused by *Blumeria* (=*Erysiphae*) *graminis*. Symptoms of powdery mildew disease include chlorotic or necrotic leaves, and stems and fruits covered with mycelium and fruiting bodies of the fungus, often giving a white 'woolly' appearance.